COMPARATIVE SERUM PHTHALATE CONCENTRATIONS IN FERTILE VERSUS INFERTILE MEN AND WOMEN IN SASKATCHEWAN

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Saskatoon

By

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ABSTRACT

Objective: To determine whether serum phthalate concentrations differ in men and women with infertility compared to those without infertility in Saskatchewan

Hypothesis: Serum phthalate concentrations will be greater in men and women with infertility compared to fertile men and women

Setting: Patients undergoing assisted reproduction for the treatment of infertility; healthy volunteers recruited from the community

Recruitment and sample collection: Infertile couples were recruited prior to in vitro fertilization (IVF) therapy for treatment of unexplained infertility (n=15), polycystic ovarian syndrome (PCOS, n=13), and male factor infertility (n=12); fertile men (n=15) and women (n=15) were recruited using poster advertisements**.** Blood samples were collected by venipuncture for phthalate analysis.

Main outcome measures: Serum phthalates concentrations (ng/mL)

Design: Prospective cohort pilot study

Methods: In infertile couples, blood samples were collected on the following 3 days of the IVF cycle: early during ovarian stimulation, day of oocyte retrieval and day of embryo transfer. In healthy volunteers, 3 blood samples were collected over a 2 week period. Liquid chromatography and tandem mass spectrometry (LC-MS/MS) was conducted to quantify concentrations of four phthalates: di-*n*-butyl phthalate (DBP), diethyl phthalate (DEP), di-(2-ethylhexyl) phthalate (DEHP) and diisodecyl phthalate (DIDP). Phthalate concentrations were compared among the four study groups using non-parametric Kruskal-Wallis and Mann-Whitney U post hoc tests.

Results: Serum DEHP and DEP concentrations did not differ among control, unexplained, PCOS, and male factor infertility groups in both men and women ($p>0.05$). DBP in women did not differ among study groups ($p=0.205$). In contrast, DBP was lesser in men with unexplained, PCOS, and male factor infertility compared to controls ($p < 0.05$). Similarly, DIDP was lesser in women of couples with unexplained, PCOS and male factor infertility groups compared to fertile women ($p < 0.05$). Less DIDP was detected in men with unexplained and male factor infertility compared to the control group ($p < 0.05$)

Conclusion: Serum phthalate concentrations in serum were lesser or not different in infertility patients undergoing IVF compared to fertile volunteers. These findings do not support the

notion that serum phthalate concentrations are associated with human infertility. Further research is needed to determine whether phthalate concentration in blood cells and adipose tissue differ in infertile versus fertile men and women.
Key words: Phthalates, PCOS, Unexplained infertility, Male factor infertility

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DEDICATION

This thesis is dedicated to my beloved father,

Professor Prokash Chandra Karmoker (belated)

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
AF	Amniotic fluid
AGD	Anogenital distance
AI	Analyte intensity
APA	Analyte peak area
APCI	Atmospheric pressure chemical ionization
APPI	Atmospheric pressure photoionization
AR	Androgen receptors
ASC1	Androgen receptors co-activator
ATSDR	Agency for Toxic Substances and Disease Registry
BBP	Benzyl-butyl phthalate
BBzP	Butyl-benzyl phthalate
BC	Blood cells
BFB	Blood-follicle barrier
BFRs	Polybrominated flame retardants
BMP-15	Bone morphogenetic protein-15
BMP-6	Bone morphogenetic protein-6
BPA	Bisphenol A
BS	Blood serum
BTB	Blood-testis barrier
cAMP	Cyclic adenosine monophosphate
CASA	Computer-aided sperm analysis
CDC	Centers for disease control and prevention
CI	Chemical ionization
CID	Collision induced disassociation
CL	Corpus luteum
CMTA	Critical mechanism of toxicity
CYP17	Cytochrome P450c17
DBP	Di-*n*-butyl phthalate
DEHP	Di-(2-ethylhexyl) phthalate
DEP	Diethylphthalate
DF	Dominant follicle
DHEAS	Dehydroepiandrosterone sulfate
DIBP	Diisobutyl phthalate
DIDP	Diisodecyl phthalate
DINP	Diisononyl phthalate
DIOP	Diisooctyl phthalate
DMP	Dimethyl phthalate
DNA	Deoxyribonucleic acid
DnNP	Di-*n*-nonyl phthalate
DnOP	Di-*n*-octyl phthalate
DnPP	Di-*n*-pentyl phthalate

E_2	Estradiol
EDC	Endocrine disrupting chemicals
EPA	Environmental Protection Agency
ER	Estrogen receptors
ESI	Electrospray ionization
FABP	Fatty acid binding protein
FAI	Free androgen index
FF	Follicular fluid
FSH	Follicle stimulating hormone
GC	Granulosa cell
GC-MS	Gas chromatography and tandem mass spectrometry
GDF-9	Growth differentiation factor-9
GnRH	Gonadotropin releasing hormone
HMW	High molecular weight
HOX	Homeobox gene
HPG	Hypothalamic-pituitary-gonadal
HPLC	High performance liquid chromatography
InsI3	Insulin-like factor 3
IOI	Interovulatory interval
IS	Internal standard
kg	Kilogram
Kow	Octanol-water partition coefficient
LC-MS-MS	Liquid chromatography and tandem mass spectrometry
LH	Luteinizing hormone
LIN	Linearity
LLE	Liquid-liquid extraction
LMW	Low molecular weight
LOAEL	Lowest-observed-adverse-effect level
LOD	Limit of detection
m/z	Mass to charge ratio
MBP	Mono-butyl phthalate
MBzP	Mono-benzyl phthalate
MDL	Method detection limit
MEHHP	Mono-(2-ethyl-5-hydroxyhexyl) phthalate
MEHP	Mono-(2-ethylhexyl) phthalate
MEOHP	Mono (2-ethyl-5oxohexyl) phthalate
MEP	Mono-ethyl phthalate
MF	Male factor
mg	Milligram
MiBP	Mono- isobutyl phthalate
min	Minute
MiNP	Mono- isononyl phthalate
mL	Milliliter

mm	Millimeter
MMP	Mono-methyl phthalate
MnBP	Mono-*n*-butyl phthalate
MOA	Mechanism of action
MRM	Multiple reactions monitoring
mRNA	Messenger ribonucleic acid
MTBE	Methyl *tert*-butyl ether
ng	Nanogram
NHANES	National health and nutrition examination survey
NOAEL	No-observed-adverse-effect level
P450scc	Cytochrome P450 side-chain cleavage enzyme
PCBs	Polychlorinated biphenyls
PCOS	Polycystic ovarian syndrome
PCP	Personal care products
$PGF_{2\alpha}$	Prostaglandin-$F_{2\alpha}$
POF	Premature ovarian failure
POPs	Persistent organic pollutants
PPARs	Peroxisome proliferator- activated receptors
PVC	Polyvinyl chloride
QA	Quality assurance
QC	Quality control
R^2	Regression coefficient
RfD	Reference doses
RNA	Ribonucleic acid
RT	Retention time
SCJP	Sertoli cell junctional protein
sec	Second
SHBG	Sex hormone binding globulin
SP	Seminal plasma
SPE	Solid phase extraction
SRB1	Scavenger receptor class B-1
ST	Seminiferous tubules
StAR	Steroidogenic acute regulator protein
TDS	Testicular dysgenesis syndrome
TOF	Time of flight
U of S	University of Saskatchewan
VCL	Curvilinear velocity
VSL	Straight-line velocity
µg	Microgram
µL	Microliter
17β-HSD-IV	17β-hydroxysteroid dehydrogenase-IV
2cx-MMHP	Mono-[2-(carboxymethyl) hexyl] phthalate
3β-HSD	3β -hydroxysteroid dehydrogenase
5cx-MEPP	Mono-(2-ethyl-5-carboxypentyl) phthalate

CHAPTER 1: GENERAL INTRODUCTION

The human endocrine system is highly complex. The main functions of the endocrine system are to synthesize and release hormones, which regulate the growth, development, and metabolism of organs and body systems. In addition to endogenous endocrine hormones, many chemicals in the environment can affect endocrine function. Endocrine disrupting chemicals (EDCs) are defined as exogenous agents that interfere with the synthesis, secretion, transport, binding, action and/or elimination of natural hormones responsible for the maintenance of homeostasis, reproduction, development and behavior (1). Endocrine disrupting chemicals can mimic or interfere with the action of endogenous endocrine hormones, including reproductive, thyroid, adrenal and pituitary hormones.

The relationship between EDCs and health has been investigated in animals and to a lesser extent in humans over the past 50 years. A growing body of evidence suggests that EDCs may be associated with a wide range of diseases and disabilities including obesity, diabetes, cancer, heart disease, thyroid disease, reproductive dysfunction, as well as neurodevelopmental and neurodegenerative disorders (2). Reproductive dysfunction and abnormal fetal development have been shown to be related to EDC exposure in many animals (3-5). Endocrine disrupting chemicals may exert their effects by a number of mechanisms including: 1) acting as estrogen agonist or aryl hydrocarbon receptor agonist, 2) acting as estrogen or androgen antagonist, 3) altering steroidogenic enzyme gene expression, 4) disrupting gonadotropin hormone production, 5) indirectly interfering with steroidogenesis by alteration of cell membrane ion exchange, 6) inducing epigenetic changes, and/or 7) inducing oxidative stress (6-11). In addition, direct effects on gametes (i.e., oocytes, sperm) have been observed (12).

Hundreds of natural and man-made EDCs are present in the environment. More than 500 chemicals are known or suspected to have endocrine disrupting potential (13, 14). Bisphenol A (BPA) and phthalates are 2 groups of man-made EDCs of particular concern because of their high production, widespread use and omnipresence (5). Women, men and children are continuously exposed to BPA and phthalates through contaminants in the air, water, soil, dust, construction materials, children's products, personal care products (e.g., perfumes, lotions and cosmetics), paints, medical devices, pharmaceuticals and many more. Phthalates are a family of industrial compounds with a common chemical structure, dialkyl

or alkyl/aryl esters of 1, 2-benzenedicarboxylic acid. Phthalates are primarily used to increase the flexibility, transparency, durability and longevity of plastics (15, 16). As additives, phthalates are not covalently bound to plastic polymers. Because phthalates easily leach from their matrix into the soil and water, and eventually gas off into the air, it is possible for phthalates to easily enter into the food chain (15, 16). Worldwide, more than 8.2 million tons of phthalates are used every year. Di-(2-ethylhexyl) phthalate (DEHP) is the most commonly used in manufacturing. It is estimated that 2 million tons of DEHP is produced each year (15-19).

Numerous biological effects have been attributed to phthalates. Phthalates have been found to cause embryo toxicity, teratogenicity, developmental anomalies, and reproductive toxicity in animals (20-23). Phthalates, particularly di-*n*-butyl phthalate (DBP) and DEHP have been more likely to cause reproductive toxicity and developmental abnormalities in animals (23-26). Anti-androgenic effects, such as short anogenital distance (AGD), cryptorchidism, decreased testosterone levels and decreased sperm production have been reported after exposure in-utero (23-26). DEHP and its bioactive metabolite, mono-(2-ethylhexyl) phthalate (MEHP), prolonged the estrous cycle, inhibited ovulation and decreased systemic estradiol concentration in cycling rats, and were thereby considered to elicit toxic effects in the ovary (27, 28). Phthalates have also been considered as obesogens (29). Mechanisms for phthalate-induced obesity include activation of peroxisome proliferator-activated receptors (PPARs), anti-thyroid activity, and anti-androgenic activity. Childhood obesity has been linked with low dose exposure, particularly during fetal life (29).

Most research about the biological effects of phthalates has been conducted in animal models; there have been few studies regarding human effects. Evidence for reproductive dysfunction in animals following exposure to BPA is available, but human studies are limited. In laboratory animals, prenatal exposure to BPA at doses consistent with human environmental exposure has been associated with inhibition of folliculogenesis and ovulation, recurrent miscarriage, ovarian cysts, oviductal lesions, endometrial hyperplasia, uterine polyps, cervical sarcoma, mammary adenocarcinoma and changes in adult brain structure, chemistry and behavior (5, 10, 30). In animals, BPA has been active as both an agonist and a competitive inhibitor of endogenous estrogen receptors, and as an antagonist of androgen receptors (31-34).

Both BPA and phthalates have been shown to affect reproduction in many animal studies. Similar research to investigate the reproductive health risks in men and women has been sparse. Prenatal exposure to BPA has been implicated as a cause of impaired fetal testicular function and a decrease in local androgen production in men as evidenced by the development of a short AGD and incomplete testicular descent (35). In addition, correlations between urinary phthalate metabolites, abnormal semen quality and decreased steroid hormone levels in men have been reported (15, 36-38). Metabolites of phthalates and BPA have been detected in human body fluids such as urine, serum, breast milk, saliva, sweat, ovarian follicular fluid (FF), seminal plasma (SP), peritoneal fluid and amniotic fluid (AF) (3, 39-48). Greater concentrations of BPA and phthalates and/or their metabolites were detected in men and women with reproductive dysfunction compared to fertile men and women (18, 39, 49); however, continued research is required to confirm these preliminary findings.

A decline in reproductive health has been observed in men and women within industrialized countries; the reasons for the decline are not well known. The worrisome changes have been demonstrated by a greater frequency of abnormal semen parameters, infertility and testicular cancer in men; an increased incidence of congenital abnormalities of the reproductive tract in both men and women; and a decline in the pregnancy and birth rates, ovarian dysfunction, and an early onset of puberty and menopause in women (50-54). The current prevalence of infertility in Canada ranges from 11.5% to 15.7%, and is similar to that of other industrialized countries (55, 56). The underlying pathophysiology for many etiologies of infertility is not fully understood. Infertility is diagnosed when a couple fails to conceive within 12 months. Unexplained infertility is diagnosed in 15%-30% of infertile couples when there is no identifiable cause. Because of the association between reproductive dysfunction in animals exposed to EDC, there has been concern that EDC are the causal agents for infertility in humans.

Research to elucidate the potential effects of EDCs, in particular phthalates, on human reproductive health are needed. Studies conducted to date have been limited by small samples sizes, differences in methodologies, and high background contamination levels leading to contradictory results and inconclusive findings. There is paucity of research to investigate the relationship between phthalates and human fertility. To date, there has been no study conducted to quantify phthalates in body fluids of men and women within Saskatchewan. Data from a pilot

study in our lab have suggested that serum BPA concentration were greater in male and/or female partners of couples diagnosed with unexplained infertility compared to controls; similarly, phthalate concentrations in the cellular component of blood were greater in women with Polycystic Ovary Syndrome (PCOS) compared to fertile women (Baerwald *et al.*, unpublished data). Further investigations are needed to confirm these preliminary findings.

In the following chapters, graduate research to compare phthalate concentrations in fertile versus infertile men and women is described. A literature review has been completed to discuss male and female reproductive anatomy, histology, and physiology; reproductive dysfunction; physiochemical properties, toxicokinetics and toxicodynamic effects of EDCs (in animals and humans); as well as analytical methods for phthalates quantification. The rationale and hypothesis for the proposed research are followed by the study methodology, results and conclusions.

CHAPTER 2: LITERATURE REVIEW

2.1 Human Reproductive Anatomy

2.1.1 Female Reproductive System

The female reproductive system consists of internal reproductive organs including the uterus, ovaries (female gonads), fallopian tubes (oviducts/uterine tubes), and external reproductive organs including the vagina and pudendum. More detailed descriptions of the reproductive organs are included below.

2.1.1.1 Ovaries

The ovaries are the female gonads. The ovaries are situated on either side of the uterus in the ovarian fossa of the female pelvis (57). During embryonic development the ovaries form alongside the primitive kidneys at the thoracic level and descend to the brim of the pelvic cavity by the third month of intrauterine life. A number of ligaments maintain the position of each of the ovaries. The mesovarium is a double fold of parietal peritoneum connecting each ovary to the right and left sides of the uterus (medially) and to the pelvic walls (laterally). The medial connector between the uterus and the ovary is called the ovarian ligament. The suspensory ligament is the lateral anchor of each ovary to the pelvic wall. Each of the ovaries is comprised of four parts, from outer to inner: 1) the germinal epithelium, a layer of cuboidal epithelium covering the surface of the ovary; 2) the tunica albuginea, a dense connective tissue layer; 3) the cortex, the outer connective tissue layer containing the different stages of growing follicles; and 4) the medulla, the inner connective tissue layer containing the blood vessels, nerves, smooth muscles, and lymphatics (57). The ovarian hilum is the space through which blood vessels and nerves enter and exit the ovary.

The blood-follicle barrier (BFB) is found within developing follicles of the mammalian ovary. The barrier separates the avascular granulosa cell layer from the vascular thecal cell layer that surrounds each follicle. The barrier is comprised of tight junctions between endothelial cells of the capillaries and the basement membrane of granulosa cells of the developing follicles (58). The BFB alters its composition rapidly during follicle development; changes in both size and charge selectivity occur (59, 60). The barrier acts as a molecular sieve to create a specialized

environment within growing follicles. The transfer of serum proteins from the blood into follicles during follicular development is regulated by the BFB (60, 61).

2.1.1.2 Uterus and Cervix

The uterus has many functions as a reproductive organ. The uterus transports sperm from the vagina to the uterine tube, and is the site of embryonic implantation, fetal development, and parturition (57). Anatomically, the uterus has three parts from upper to lower: 1) the fundus, the upper dome shaped part above the uterine cavity; 2) the body with a tapering central portion with a triangular cavity; and 3) the cervix, the lower tubular portion which connects the uterus to the vagina (57). Histologically, the uterus has three layers from outer to inner: 1) the perimetrium, a part of visceral peritoneum forming a vesico-uterine pouch anteriorly and recto-uterine pouch posteriorly; 2) the myometrium, consisting of a middle circular and outer and inner longitudinal smooth muscles fibers; and 3) the endometrium, containing an epithelium, stroma, and glands (57). Endometrium is arranged in two layers. The inner most layer of the endometrium is the stratum functionalis, which undergoes proliferation and loss via menstruation. The deeper layer of endometrium is called the stratum basalis. The basal layer of endometrium is maintained during menses and regenerates the functional layer following menses (62, 63). During embryological development, the Müllerian ducts gives rise to bilateral fallopian tubes, a single uterus and upper vagina.

2.1.1.3 Fallopian Tubes

A fallopian tube (also referred to as oviduct) extends from the right and left horns of the uterus to each ovary. The function of the oviducts is to facilitate the transport of sperm to ovum and embryo to uterus (57). Each oviduct is approximately 10 cm in length and has three parts from medial to lateral: 1) isthmus extends from the uterine horn to the ampulla; 2) ampulla, the the usual site for fertilization; and 3) infundibulum, contains fimbriae distally which assist with transport of the ovum from the ovary into the oviduct for fertilization (57).

2.1.2 The Male Reproductive System

The male reproductive system consists of internal and external genital organs. The internal genital organs are: 1) the testes, which produce sperm and secret male sex hormones; 2)

the duct system including the epididymis, ductus deferens, ejaculatory duct and urethra, which are required for the storage, maturation, and transport of sperm; and 3) the accessory sex glands including the seminal vesicles, prostate, and bulbourethral glands, whose secretions contribute to semen (57). The external genital organs are: 1) the scrotum, which provides support for the testes and maintains the temperature for spermatogenesis; and 2) the penis, that delivers sperm into the female vagina (57).

2.1.2.1 Testes

The testes are the primary reproductive organs. Histologically, each testis consists of the following layers from outer to inner: 1) tunica vaginalis, the outer peritoneal layer; 2) tunica albuginea, the inner visceral peritoneal layer that divides the testis into 200-300 lobules; 3) parenchyma, which consists of the seminiferous tubules (ST), interstitial cells of Leydig, connective tissue, capillaries and lymphatic vessels; and 4) hilum, a space which accommodates the rete testes, an anastomosing network of delicate collecting tubules (57). Tight junctions between adjacent sertoli cells and peritubular capillary endothelial cells surround the ST to create the blood-testis barrier (BTB). The BTB is designed to prevent destruction of the developing germ cells by harmful substances such as antigens, antibodies, and environmental toxins (64, 65).

2.2 Human Reproductive Physiology

2.2.1 Female Reproduction

Female reproductive physiology involves ovarian and uterine cycles, which are highly coordinated. The main function of the ovary is to cyclically release oocytes (i.e., eggs) and produce steroid hormones through the processes of follicle growth, maturation and ovulation (57). Ovulation is the release of the oocyte from a preovulatory follicle into the peritoneal cavity. The oocyte is taken up into the fallopian tube for fertilization. Following ovulation, the follicle transforms into the corpus luteum (CL), a gland responsible for hormone production and maintaining early pregnancy.

In concert with the ovarian cycle, the uterine cycle involves proliferation and differentiation of the endometrium. The menstrual cycle is under the influence of cyclic changes

in ovarian hormones. The endometrium develops in preparation for implantation of an embryo. In the absence of pregnancy, ovarian hormonal support of the endometrium is withdrawn and dissolution of the endometrium culminates in menstruation. The endometrial layers slough off during menstruation and the cycle ends. The length of each menstrual cycle varies from 23-35 days and averages 28 days.

2.2.1.1 The Ovarian Cycle

2.2.1.1.1 Follicular Dynamics

Folliculogenesis is the process of growth, maturation and regression of follicles within the ovaries. This process starts at approximately 24 weeks of gestation and ends at menopause (66, 67). After initiation, the duration of follicular growth from primordial follicle stage to onwards takes approximately 175 days. Folliculogenesis can be divided into pre-antral and antral stages. The developmental transition from a resting state to a growing preantral follicle occurs over approximately 3 months (66, 67). Development to a mature pre-ovulatory antral follicle occurs over the subsequent 85 days. Pre-antral follicle development occurs independent of pituitary gonadotropins whereas antral follicle development is regulated, following puberty, by the cyclic production of gonadotropins (67, 68). Pre-antral follicle development is divided into primordial, primary, and secondary stages. Antral follicle development stages are described as early, mid, late and preovulatory.

Folliculogenesis is preceded embryologically by the period of oogenesis, the formation of female gametes. Between 4 to 6 weeks gestation, primordial germ cells (PGC) migrate from the dorsal wall of the yolk sac to the primitive indifferent gonadal ridge of the embryo (69, 70). In the absence of male hormones, PGCs undergo mitotic division. Differentiation of PGC into oogonia occurs with the onset of meiotic cell division. Oogonia development is arrested at prophase-I of the first meiotic division; oocytes at this stage of growth are called primary oocytes (57). Most oogonia undergo degeneration at early stages of development, contributing to depletion of the ovarian reserve. Primary oocytes remain arrested at diplotene stage of meiosis I until the onset of puberty, at which time gonadotropic hormone production mediates the cyclic process of folliculogenesis (57). At the time of ovulation, the primary oocyte completes the

second meiotic division, and is termed a secondary oocyte. The second meiotic division is completed at the time of fertilization.

2.2.1.1.1.1 Pre-antral Folliculogenesis

Pre-antral follicle development involves the transition from primary to secondary and early tertiary follicles. Stages of folliculogenesis are identified by the number of layers of granulosa cells, development of theca cells and the appearance of fluid filled cavity (antrum) (68). At the beginning of pre-antral development, primordial follicles are approximately 35-38 μm in diameter and contain a single layer of squamous-derived granulosa cells around the primary oocyte (71). The mechanism underlying the initiation of primordial follicle growth and development is not well understood (71). Primordial follicles develop into primary follicles measuring approximately 46μm in diameter, which contain a single layer of cuboidal granulosa cells. At the primary follicle stage, a mucopolysaccharide membrane called the zona pellucida surrounds the oocyte (57, 72). Primary follicles develop into secondary follicles, which are 80-100 μm in diameter and contain two or more layers of granulosa cells with an independent blood supply (67). Secondary follicles begin to express follicle stimulating hormone (FSH), estradiol (E_2) and androgen receptors (AR) (67). As the follicle enlarges, ovarian stromal tissues also stratify and differentiate into theca externa and interna. Preantral follicles (150 μm) contain a steroid secreting theca interna layer. Preantral follicles are sensitive to gonadotropins; however, their growth and development is thought to be mediated mainly by oocyte-derived factors (i.e., growth differentiation factor-9 (GDF-9), bone morphogenetic protein-15 (BMP-15), and granulosa cell-derived factors (i.e., anti mullerian hormone) (73-76).

2.2.1.1.1.2 Antral Folliculogenesis

The later stages of folliculogenesis include the development of early antral, antral and preovulatory (also known as “Graafian”) follicles. The antrum is a fluid-filled cavity that develops within the follicle (67, 71, 77). Early antral follicles are approximately 0.2 to 2 mm in diameter. Cyclic recruitment of early antral follicles begins at the time of puberty when the hypothalamic-pituitary-ovarian axis is activated. Antral follicles of 2-5 mm size can be detected ultrasonographically or histologically at any stage of the menstrual cycle during reproductive life (77-79). Antral follicles are dependent upon gonadotropins for growth and ovulation.

Follicular Recruitment: Follicular recruitment (also referred to as "wave emergence") is defined as the synchronous growth of a group or cohort of antral follicles. The pattern of cyclical follicular recruitment has been debated for a long time (78, 80, 81). Three different theories of follicular recruitment have been proposed: 1) continuous recruitment throughout reproductive life (82-84); 2) recruitment of a single cohort of antral follicles during the late luteal or early follicular phase (84, 85); and 3) recruitment of two or more waves of antral follicles in each menstrual cycle (78, 84, 86, 87). Because antral follicular waves have been observed in animal species and women, the wave theory has emerged as the most acceptable theory to date. Two and three waves of follicles have been demonstrated in menstrual cycles of women at all reproductive ages (88). The length of the menstrual cycle has been positively associated with the number of waves that developed (88). Follicle waves have been described as either major or minor. Major waves are those in which a single "dominant" follicle is selected for preferential growth. A dominant follicle within a major wave may continue growth and development to ovulation or may regress as an anovulatory follicle. Minor waves are those in which a dominant follicle is not selected (84). A rise in FSH is thought to induce the emergence of each follicular wave.

Ovarian follicular wave dynamics are studied over an interovulatory interval (IOI) rather than a menstrual cycle, in order to maintain methodological consistency among animal and human studies. An IOI is the period from one ovulation to the subsequent ovulation; it is comprised of one luteal phase followed by one follicular phase. Thus, an IOI is an inverted menstrual cycle. In women of reproductive age, the final wave of an IOI occurs within the follicular phase and culminates with ovulation (termed a major ovulatory wave). All preceding waves are either major or minor anovulatory waves (84). The length of the IOI has been positively correlated with the number of waves that develop (88). The luteal phase major wave illustrated in Figure 2.2.1 occurs in approximately 15% of women with 2-wave patterns of follicle development (78, 84). Luteal phase major waves (i.e., major-minor-major or major-major-major) occur in approximately 38% of women with 3-wave patterns of follicle development (Figure 2.2.2) (78, 84).

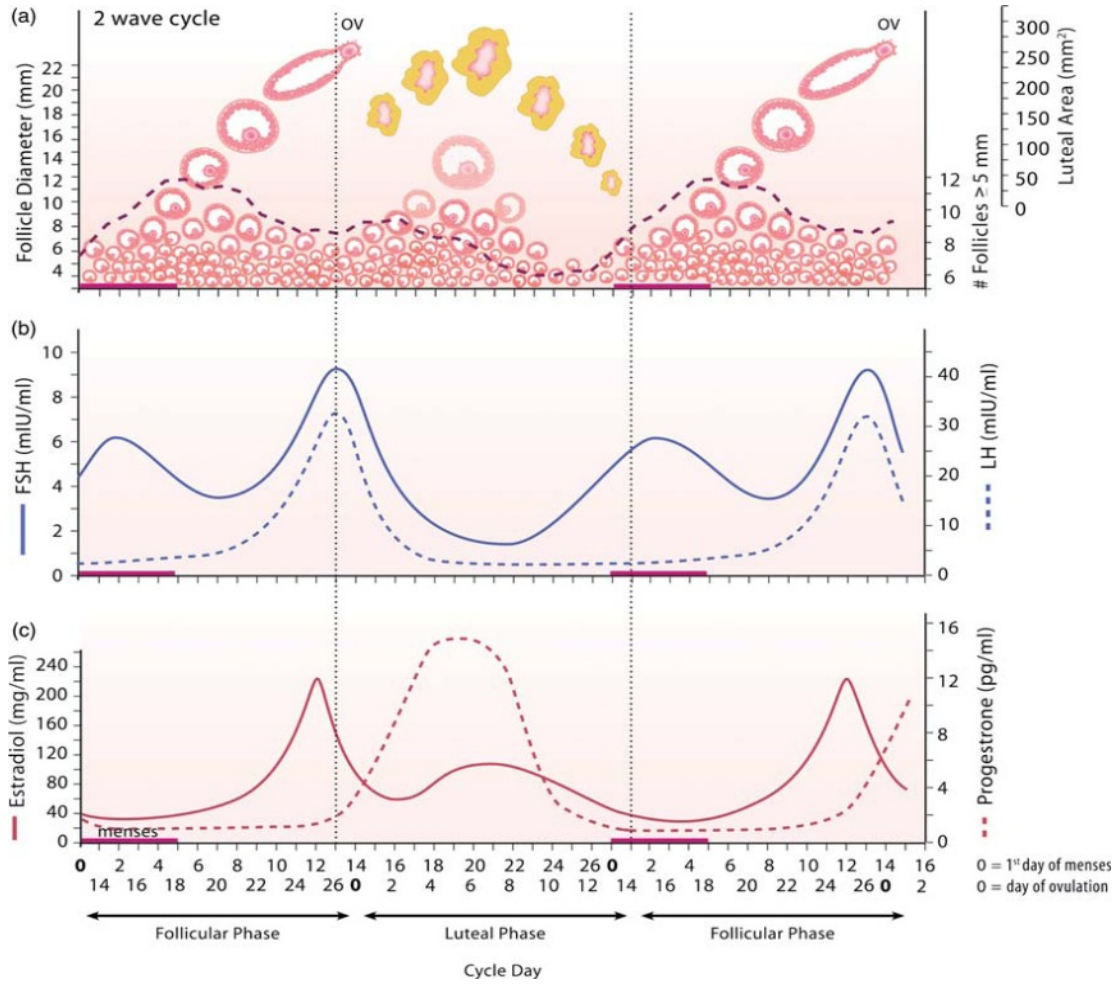

Figure 2.2.1: Antral folliculogenesis and corresponding changes in reproductive hormones during 2 waves of follicle development. Two waves of follicle development occur in approximately 68% of reproductive-aged women. One menstrual cycle is represented by a follicular phase and a luteal phase. One full interovulatory interval (IOI) is comprised of one luteal phase and one follicular phase. Dotted vertical lines indicate wave emergence. Used with permission from Blackwell Publishing Ltd., Human Reproduction Update, 2012 (84).

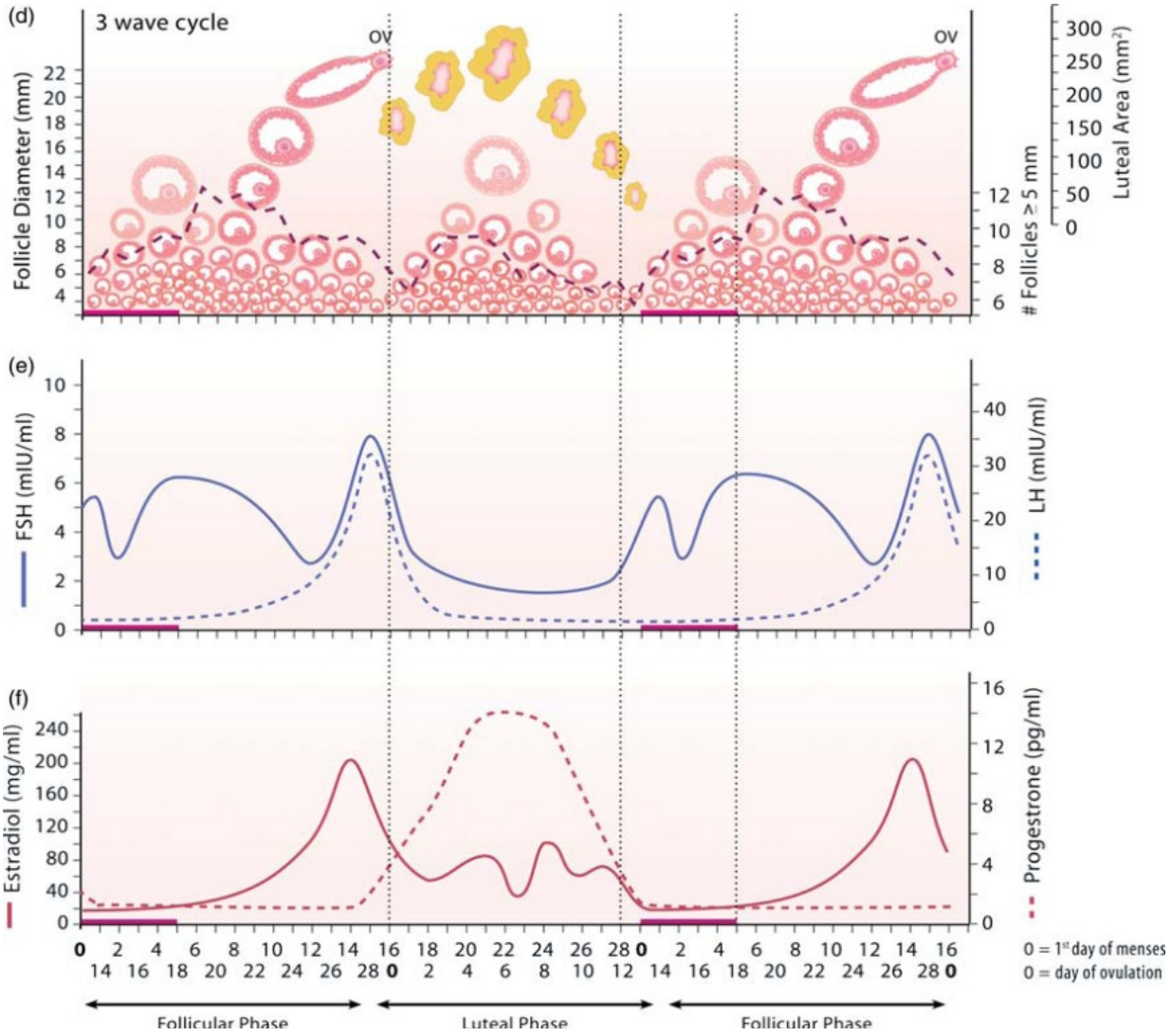

Figure 2.2.2: Antral folliculogenesis and corresponding changes in reproductive hormones during 3 waves of follicle development. Three waves of follicle development occur in approximately 32% of reproductive-aged women. One full menstrual cycle (i.e. the follicular phase and luteal phase) and one full IOI (i.e. the luteal phase and follicular phase) are shown. Dotted vertical lines indicate wave emergence. Used with permission from Blackwell Publishing Ltd., Human Reproduction Update, 2012 (84).

Follicle Selection: Selection of a dominant follicle is described as the preferential growth of a follicle from a wave of growing follicles. Within a given major wave, a single follicle is typically selected. However, in some waves (especially in women during the transition to menopause) more than one dominant follicle may be selected (89). The number of dominant follicles selected appears to depend upon the duration of rise of FSH, referred to as the "FSH window" (84). Non dominant follicles within a wave are termed "subordinate" follicles (84, 90).

The growth of dominant follicles is highly dependent upon hormone production. Inhibin B is produced by all follicles within a wave. Inhibin B exerts negative feedback on FSH secretion from the anterior pituitary (91-93). Selection of the dominant follicle occurs following a shift from FSH to luteinizing hormone (LH) receptor expression on granulosa cells. At the time of selection, the dominant follicle expresses aromatase and produces 17-B estradiol. Subordinate follicles, in contrast, are androgenic. The dominant follicle has been shown to contribute 90% of systemic estradiol during the mid-late follicular phase of the human menstrual cycle (94). Secretion of insulin-like growth factor-1 and inhibin A also facilitate preferential growth of the dominant follicle in the mid-late follicular phase (95-97).

Preovulatory Growth and Ovulation: The dominant follicle undergoes accelerated growth at a rate of 1-4 mm/day prior to ovulation (67, 78, 95). A preovulatory follicle attains a diameter of 16-28 mm. Preovulatory follicle growth is regulated by LH, inhibin A, and oocyte-derived growth factors, such as GDF-9, BMP-15 and BMP-6 (76, 98, 99). In the late follicular phase, estradiol from the dominant follicle provides positive feedback to the hypothalamus and pituitary to induce an LH surge (93). The LH surge is the stimulus for ovulation. The LH surge induces the primary oocyte to complete meiosis-I to become a secondary oocyte (93). Ovulation occurs approximately 36 hours following the onset of the LH surge (100).

In addition to inducing oocyte maturation, the LH surge stimulates a cascade of reactions within the preovulatory follicle. LH induces granulosa cell prostaglandin E_2 and $F_{2\alpha}$ production, which increases blood flow in the preovulatory follicle (89, 100). Luteinizing hormone stimulates the release of histamine in the preeovulatory follicle, which increases capillary permeability in the follicle wall (101). Vasodilation and increased capillary permeability result in an increase in intrafollicular pressure. The LH surge also induces proteolytic enzymes that degrade the follicular wall (100, 102). Breakdown of the tunica albuginea and the supporting

epithelial basement membrane result in expansion of the granulosa cells surrounding the oocyte and release of the oocyte into the follicular antrum (100, 102). The process of cellular death within the wall of the preovulatory follicle includes both apoptosis and necrosis. The apical wall of the preovulatory follicle thins while the intrafollicular pressure increases. The ultimate result of the coordinated morphologic, physiologic and biochemical event is ovulation, the rupture of the pre-ovulatory follicle and release of the oocyte. After ovulation, the oocyte is taken up by the fimbriae of the fallopian tube in anticipation of conception (61, 100). Ovulation signals the onset of the luteal phase of the menstrual cycle.

2.2.1.1.2 Luteal Dynamics:

After ovulation, cells from the pre-ovulatory follicle undergo morphological and functional changes. Granulosa cells of the preovulatory follicle transform into large cells called granulosa lutein cells. Adjacent theca cells transform into small cells called theca lutein cells (103-105). The granulosa and theca lutein cells comprise the corpus luteum.

The luteal phase is characterized by luteal growth and regression as well as progesterone synthesis. Both granulosa and theca lutein cells produce progesterone, and to a lesser extent estradiol, androgens and inhibin. After ovulation, rapid vascularization of luteal cells contributes to increased endocrine hormone production (104). LH stimulates the conversion of pregnenolone to progesterone in granulosa and theca lutein cells, through the activation of 3β-hydroxysteroid dehydrogenase (3β-HSD) enzyme (106). Granulosa lutein cells secrete both progesterone and estrogen (76, 107). The diameter, vascularity and progesterone production of the CL peak around 7 days post ovulation in non-conception menstrual cycles. Progesterone maintains the stability of endometrium and increases the glandular activity of endometrium to provide an environment supportive for implantation and conception. The role of luteal estradiol production is not known.

In the absence of conception, regression of the CL occurs during a process referred to as luteolysis (108). Luteolysis begins approximately 6-7 days after ovulation. Prostaglandin $F_{2\alpha}$ (PG $F_{2\alpha}$), a vasoconstrictor produced by the CL, is the main trigger for the luteolysis (108, 109). $PGF_{2\alpha}$ causes vasoconstriction and reduced blood flow to the CL. Regression of the CL culminates in a decline in progesterone and estrogen secretion and regression of the luteal phase.

As progesterone decreases, the negative feedback exerted by progesterone and estrogen is withdrawn, FSH rises, and the next menstrual cycle begins (107, 110). The reduction of progesterone and estradiol in luteal phase induces ischemic changes in the endometrium and sloughing of the endometrial lining during menses.

2.2.1.2 The Uterine Cycle

The uterus is an endocrine organ which responds to hormonal signals from the ovary throughout the menstrual cycle in order to prepare the uterus for potential pregnancy. The cycle of endometrial growth and regression is discussed in the following main phases (62):

1) Proliferative phase

Increased estradiol secretion during the follicular phase stimulates growth and proliferation of the stratum functionalis layer. Menstruation ceases as endometrial growth begins; endometrial growth peaks just prior to ovulation. The thickness of the endometrium increases from approximately 0.5 mm after menses to at approximately 10-11 mm at ovulation (111). Estradiol stimulates mitotic activity, nuclear DNA and cytoplasmic RNA synthesis in the functional layer of the endometrium (112, 113).

2) Secretory phase

Endometrial proliferation ceases after ovulation. Progesterone attenuates the action of estrogen and mediates the decrease of luteal cell mitosis and DNA synthesis in the endometrial glands (114, 115). Endometrial glands become tortuous, the spiral vessels undergo exaggerated coiling, and glycoproteins and peptides are secreted into the endometrial cavity. The peak secretory changes occur by approximately 7 days after the onset of the LH surge in non-conception cycles, and correspond with the timing for implantation.

3) Menstrual endometrium

After luteolysis, decreased progesterone and estradiol production results in endometrial ischemia, apoptosis, tissue loss and menstruation. The stratum functionalis is shed during menses. The stratum basalis is retained and participates in endometrial regeneration during the

next menstrual cycle. Menstrual fluid is composed of autolysed stratum functionalis, inflammatory exudate, red blood cells, and proteolytic enzymes (116).

2.2.2 Male Reproduction

2.2.2.1 Spermatogenesis

Primordial germ cells proliferate and differentiate into spermatogonia during embryogenesis. Roughly 300,000 spermatogonia in each gonad remain dormant during childhood. By puberty, there are approximately 600,000,000 spermatogonia in each testis. It has been estimated that following puberty 100-200 million sperm are produced each day (117). Three phases of spermatogenesis take place in the seminiferous tubule:

1) Spermatogonia undergo mitosis.
2) Primary and secondary spermatocytes undergo meiosis I and II to ultimately produce haploid (1N) spermatids
3) Spermatids undergo morphological differentiation into sperm through a process of spermiogenesis (118).

The complete process of spermatogenesis takes approximately 70 days in humans (119). An additional 12-21 days are required for sperm to travel through the epididymis in order to be ejaculated through the vas deferens (120, 121). The actions of both Sertoli and Leydig cells are needed for spermatogenesis.

Sertoli cells are comparable to the granulosa cells of ovarian follicles with respect to the cells' supportive function for male gametes. Sertoli cells extend from the basement membrane of the epithelium of seminiferous tubule to the apical lumen. Sertoli cells support and protect the developing spermatogenic cells. Sertoli cells secrete androgen binding protein, which binds testosterone and maintains it at high concentrations within the testes. The inhibin, secreted by Sertoli cells, suppresses FSH secretion from the anterior pituitary. Sertoli cells secrete fluid necessary for sperm transport.

Clusters of Leydig cells are located between adjacent seminiferous tubules. LH secreted from the anterior pituitary induces testosterone secretion from the Leydig cells (Figure 2.2.3). Testosterone is converted to dihydrotestosterone (DHT) by 5α-reductase in the Sertoli cell (118).

DHT is the biologically active form of testosterone. During embryonic development, testosterone stimulates differentiation of the Wolffian ducts into the vas deferens, epididymis and seminal vesicles, and promotes descent of the testicles into the scrotum. After conversion of testosterone to DHT, fetal external genitalia become virilized. At puberty, DHT induces enlargement of the male external and internal sex organs, as well as secondary male sexual characteristics (122).

2.2.3 Regulation of Hormone Production

The hypothalamo-pituitary-gonadal (HPG) axis plays a fundamental role in regulation of both male and female reproductive function. The gonads and anterior pituitary produce hormones and growth factors to regulate steroid hormone production in both men and women (Figure 2.2.4). The hormones provide positive and negative feedback to the pituitary and hypothalamus and affect hormone synthesis and secretion.

The HPG axis is thought to be activated temporarily during early stages of fetal developmental and then reactivated at the time of puberty. During reproductive life, gonadotropin releasing hormone (GnRH) is secreted from the arcuate and preoptic nuclei of the hypothalamus in a pulsatile manner (123). GnRH travels via the hypophyseal portal system into the anterior pituitary where it stimulates gonadotroph cells to secret the gonadotropins (i.e., FSH and LH) (124). While slow GnRH pulse frequency stimulates FSH synthesis, fast GnRH pulse frequency induces LH synthesis (125). FSH and LH bind to receptors within the ovary and testis to induce steroid production and gametogenesis.

2.2.4.1 Male Reproduction

The male HPG axis involves the hypothalamus, pituitary and testes (Figure 2.2.4). During fetal life and childhood, the release of GnRH from the hypothalamus is suppressed by an ultrasensitive negative feedback of testosterone and estradiol from the testes (126). At the onset of puberty there is decreased sensitivity of the hypothalamus to this negative feedback. After the onset of puberty, GnRH is released in a pulsatile fashion by the hypothalamus and LH and FSH are secreted by the pituitary every 2-6 hours totaling 4-8 times /day. Gonadotropin release is followed by testosterone secretion from the testis (126). LH binds to receptors on Leydig cells of the testes to stimulate the conversion of cholesterol to progesterone to testosterone.

Testosterone crosses the blood testes barrier, enters the Sertoli cells and is converted to DHT (and to a lesser degree, estrogen). FSH binds to receptors on Sertoli cells to induce the production of androgen-binding protein and inhibin-B. Because androgen binding protein binds testosterone, a high concentration of testosterone is maintained in the testes. Testosterone can then induce spermatogenesis in the testes. Inhibin-B provides negative feedback to the anterior pituitary to suppress FSH secretion (118, 124). Following inhibin-B mediated negative feedback, the LH amplitude is greater than that of FSH (126). Testosterone, DHT and estradiol from the testes also provide negative feedback control on the hypothalamus and pituitary (127).

2.2.4.2 Female Reproduction

The female HPG axis involves the hypothalamus, pituitary and ovaries (Figure 2.2.4). Before puberty, GnRH is secreted at low amplitude and low frequency from both tonic and surge centers in the hypothalamus. Low amplitude GnRH secretion is insufficient to stimulate the anterior pituitary to produce LH and FSH (126). In the prepubertal ovary, estradiol is produced at a level below the threshold level required to stimulate the estrogen sensitive surge center. After puberty onset, the tonic center is responsible for the basal secretion of GnRH. The surge center controls the large preovulatory surge of GnRH and the surge of LH by the pituitary. LH binds to receptors on theca cells located alongside follicles to stimulate the stepwise conversion of cholesterol to progesterone to androstenedione (Figure 2.2.3). Androstenedione moves from the theca cells, crosses the blood-follicle barrier and travels to the granulosa cells. FSH binds to its receptors on the granulosa cells and induces the conversion of androstenedione to estrone and testosterone to estradiol.

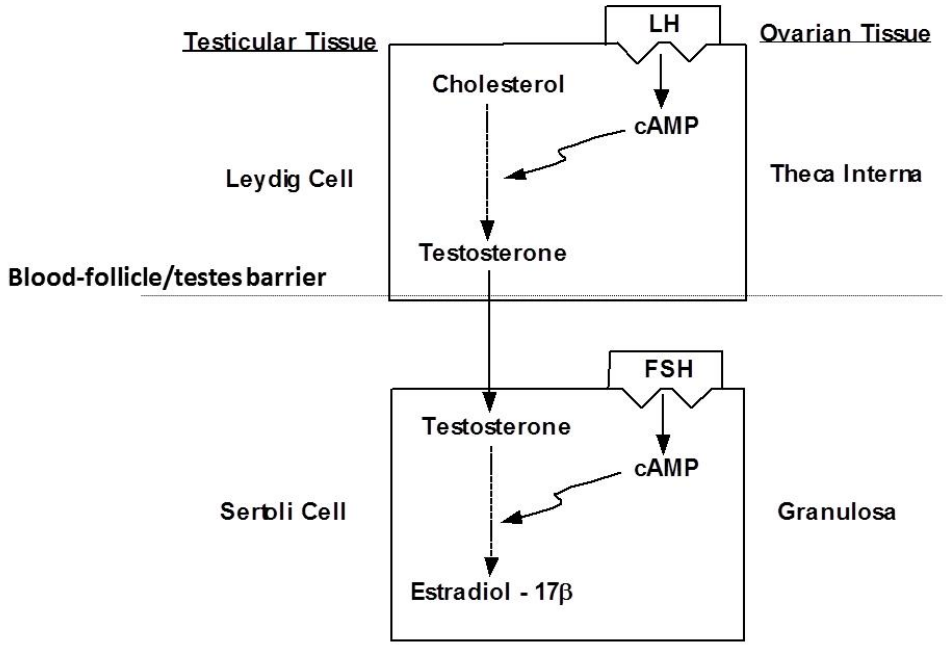

Figure 2.2.3: The 2 cell 2 gonadotropin theory of male and female reproductive function. In men, the conversion of cholesterol to testosterone takes place in Leydig cells of the testes. Testosterone migrates across the blood testes barrier into the Sertoli cells to induce spermatogenesis. In women, androgens are produced in the theca interna cells of growing follicles, and migrate across the blood-follicle barrier into the granulosa cells where they are converted to estradiol (From Baerwald 2005, unpublished).

Cyclic changes in gonadotropins regulate menstrual cyclicity. During the mid-follicular phase, inhibin B is produced by the granulosa cells of all follicles of the wave. The rising inhibin B directly inhibits the pituitary secretion of FSH (91, 92). It is thought that follicles continue to undergo atresia under the influence of declining FSH until only a single dominant follicle remains for continued growth (128). In the late follicular phase, a high concentration of estradiol, secreted by the dominant follicle granulosa cells, exerts positive feedback to the hypothalamus to induce the LH surge prior to ovulation (89, 129). During the luteal phase of the menstrual cycle, progesterone and estradiol from the CL provide negative feedback to the pituitary to suppress all but basal FSH and LH production. With regression of the CL, luteal phase progesterone and estradiol production decreases, FSH rises and the next menstrual cycle begins (82, 110).

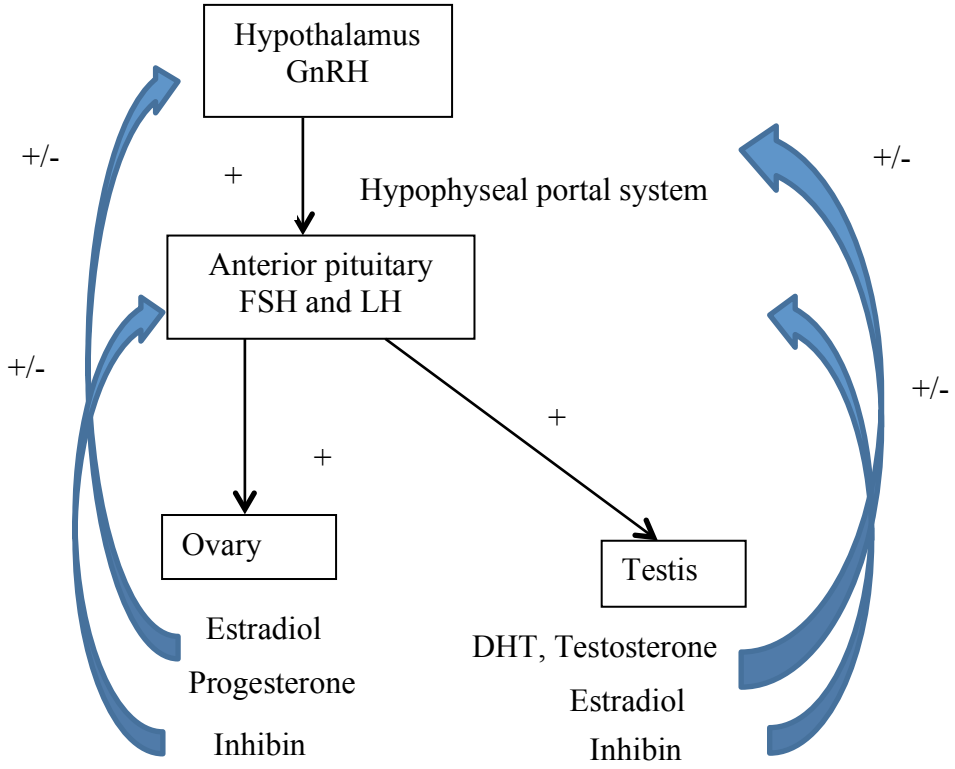

Figure 2.2.4: The hypothalamo-pituitary-gonadal axis in men and women. Gonadotropin releasing hormone (GnRH) regulates the secretion of gonadotropins (FSH and LH) from the anterior pituitary, and thereby stimulates the gonads (ovaries and testes) to produce steroid hormones. Steroid hormones provide negative (-) and/or positive (+) feedbacks at the level of hypothalamus and/or pituitary.

2.3 Reproductive Dysfunction

Many etiologies of reproductive dysfunction have been described. Approximately 1 in 6 couples experience infertility at some time in their lives (130). Infertility is defined as the inability to achieve pregnancy after one year of reasonably regular unprotected sexual intercourse (131). "Subfertility" is used to describe decreased reproductive efficiency without sterility (131). Infertility gives rise to a great deal of medical, psychosocial and economical stress for patients. Infertility can be considered primary and secondary. In primary infertility, women/couples have never conceived, whereas secondary infertility occurs after proven fertility. The etiology of infertility is roughly accounted for by male factor dysfunction in 1/3 of all

couples, female factor dysfunction in1/3, and combined male and female dysfunction in the remaining 1/3 (132).

2.3.1 Female Infertility

A dramatic change in female fertility has been observed over the past 30 years. The peak age for fertility in women is between 20 and 24 years; a progressive decrease in cycle fecundity occurs with age. The cumulative decline in fertility has been estimated to be 4-8% at 25-29 years; 15-19% at 30-34 years; 26-46% at 35-39 years; and 95% by 40-45 years (131,133, 134). Completion of advanced education, economic reasons for women to take on careers, delayed age at marriage and child bearing, and easy access to family planning services are socioeconomic factors that have contributed to a decline in both female fertility and birth rate in North America (131). Infertility may occur solely because of conditions borne by women such as ovulatory dysfunction (40%) as well as tubal and pelvic pathology (40%). "Other" miscellaneous etiologies are found in approximately 10% of women; "other" etiologies may be attributed to uterine, cervical and vaginal etiologies, advanced age, extremes of weight, smoking, excessive alcohol or drug use, occupational or environmental exposure to contaminants, and stress (131). Infertility is unexplained in 10-25% of couples.

2.3.1.1 Ovulatory dysfunction

Ovulatory disorders are one of the major reasons for female infertility. Ovulatory failure (anovulation) and infrequent ovulation (oligoovulation) are the most common presentations. Polycystic ovarian syndrome (PCOS) is a very common endocrine and metabolic disorder (135-137). Approximately 1.4 million Canadian women (12%) are diagnosed with PCOS (137, 138). Polycystic ovarian syndrome is a major cause of hormone imbalance, anovulatory infertility and miscarriage (136, 139-141). A spectrum of symptoms are seen and no single diagnostic criteria for PCOS has been identified (141, 142). The major clinical manifestations of PCOS include weight gain with central obesity, irregular and infrequent menstruation, male pattern hair distribution, acne, insulin resistance manifested as acanthosis nigricans, and mood disorders (139, 140). The diagnosis of PCOS requires at least two of the following three criteria: 1) irregular or absent menstruation (>35 days), 2) clinical and/or biochemical signs of high androgen hormone and 3) polycystic ovaries as seen ultrasonographically with exclusion of other

endocrine disorders of the adrenal, thyroid, pituitary and hypothalamus (139, 140, 142). Ultrasonographic diagnostic criterion for PCOS includes ≥ 26 antral follicles per ovary (143).

The pathogenesis of PCOS is uncertain. A combination of genetically inherited mutations (e.g., cytochrome P450c19 aromatase gene and TAAA repeats in SHBG promoter) and environmental factors may lead to excess androgen exposure during the critical intrauterine period of organogenesis (144). A fetal origin for PCOS has been proposed in rats. Fetal rats exposed to a high androgenic environment in utero expressed a PCOS phenotype and metabolic alterations later in adult life (145, 146). These studies have supported the concept that in utero exposure to a high androgenic environment may contribute to epigenetic abnormalities underlying the fetal origin and inheritance of PCOS. In women, systemic hormonal imbalances in classic PCOS include elevated LH, low FSH/LH ratio, excess androgens, low normal estradiol level without cyclic variability and high insulin (147). It is thought that insulin resistance results in greater synthesis of testosterone within the ovaries, greater production of androgens from the adrenal glands and less formation of sex hormone binding globulin (SHBG) from the liver (148). Peripheral conversion of androgens to estrogens in fat, liver, and muscle leads to low FSH and prevents follicle growth and ovulation (149, 150). Long term complications of PCOS include miscarriage, hyperlipidemia, hypertension, diabetes, cardiovascular disease and endometrial hyperplasia (139, 140, 151-153).

Premature ovarian failure (POF) is an uncommon reason for anovulation. Cessation of menstruation before the age of 40 with a low or absent ovarian antral follicle count is diagnostic of POF (154, 155). Causes of POF are most often unknown (155). The etiology may be acquired or may be of genetic origin. Acquired causes include chemotherapy, radiotherapy, surgery, autoimmune disease, infection, smoking and exposure to toxins. Genetic disorders involving the X chromosome (i.e., 45XO karyotype) have been seen in women with POF (156).

Many endocrine disorders are associated with anovulation and oligoovulation. Thyroid disease including hyper and hypothyroidism and hyperprolactinemia may culminate in anovulation (131). Adult onset congenital adrenal hyperplasia mimics PCOS as an uncommon reason for anovulation, androgen excess and hirsutism (157). Pituitary hypersecretion of prolactin may occur following the development of a pituitary adenoma or brain tumor or with

ingestion of specific medications (158). Physical and emotional stress has been associated with suppression of hypothalamic activity.

2.3.1.2 Tubal and pelvic factors

Acquired abnormalities of the reproductive organs are one of the most common reasons for infertility in women. Tubal infertility is diagnosed when the fallopian tube is congenitally abnormal or when the oviducts are structurally damaged or surgically removed (131). The most common causes of tubal infertility are obstructive processes including pelvic endometriosis, pelvic inflammatory disease, septic abortion, ectopic pregnancy, ovarian torsion and bowel infections such as ruptured appendicitis. Tubal ligation surgery causes infertility for contraceptive purposes.

2.3.1.3 Unexplained infertility

Unexplained infertility is a difficult and disconcerting diagnosis for couples. Unexplained infertility is defined by the absence of an identifiable reason for infertility. In approximately one quarter to one-third of couples, the cause of infertility is unexplained. A diagnosis of unexplained infertility is made when a couple fails to conceive within 1 year of unprotected intercourse, and all conventional diagnostic tests or methods to detect the etiology of infertility (e.g., ovulation testing, ovarian reserve, semen analysis, anatomic imaging) are normal (131, 159, 160). Abnormalities in fertilization or implantation may be observed during in vitro fertilization. Currently, no predictors for aberrant fertilization or implantation exist (161). Immunological, genetic, endocrinological and environmental factors have been proposed as potential causes of unexplained infertility (161-164).

2.3.1.4 Other factors

Other less common etiologies for female infertility have been identified. Congenital abnormalities of the uterus such as a uterine septum have been associated with recurrent first trimester miscarriage and mid–late second trimester preterm delivery (165). Other conditions that affect fertility include intrauterine adhesions (Asherman's syndrome) (165), endometrial polyps, and fibroids. Damage to the cervical canal following the growth of cervical fibroids, cervical surgery or sexually transmitted infections with chlamydia or gonorrhea may impair

sperm transport (131, 166). Anti-sperm antibodies may cause infertility by directly destroying sperm (167). Alterations of chemical signals within the HPG axis may result in infertility. Dysregulation of the HPG axis occurs with some disease conditions, such as hypothyroidism, hyperthyroidism, hyperprolactinemia and anorexia nervosa. Additional factors, such as medications, psychological and environmental stressors may also lead to hormonal changes that affect the HPG (131). Stress-mediated release of catecholamines (adrenaline, noradrenaline and dopamine) may interact with the release of GnRH, FSH, LH and prolactin production and interrupt follicle growth and ovulation (168).

2.3.2 Male Infertility

The etiology of infertility is attributed solely to the male partner in approximately 1/3 of couples. Fertility status in men has been classified as fertile, subfertile and infertile based on semen parameters (169). Infertility is considered to be of male origin when semen volume is <1 mL, sperm concentration is < 2.0 x10^6/ml, progressive motility is <10%, or normal morphology is <5%. Normal semen parameters are shown in Table 2.3.1. Reproductive dysfunction in men has been divided into congenital, acquired and idiopathic etiologies. The diagnosis is unexplained for 40-45% of men with infertility. The known causes of male infertility include hypothalamic-pituitary disorders (1-2%), primary gonadal disorders (30-40%) and disorders of sperm transport (10-20%) (170). Hypothalamic-pituitary disorders are related to infertility in patients with Kallmann syndrome, brain tumors, hyperprolactinemia, drug use, physical injury, brain infection, chronic systemic illness and obesity. Primary gonadal disorders arise from congenital conditions, such as Klinefelter syndrome, deletion of Y chromosome, various genetic mutations, crytorchidism, or acquired conditions such as testicular infection. Exposure to environmental toxins (e.g., smoking, metals, organic solvents and pesticides), drugs (e.g., alkylating agents, alcohol, antiandrogens and cimetidine), radiation, chronic systemic illnesses, and excessive heat may lead to testicular dysfunction and infertility. Disorders of sperm transport are seen with congenital absence of the vas deferens, Kartagener syndrome (a defect of cilia), epididymal obstruction or dysfunction, and blockage of vas deferens by infection from gonorrhoea, chlamydia or tuberculosis, erectile or ejaculatory dysfunction or vasectomy (170).

Table 2.3.1: Normal semen parameters (modified from WHO 5th manual, 2009-10) (171).

Volume (ml)	1.5
Sperm concentration (10^6/ml)	15
Total sperm count (10^6)	39
Motility (% progressive)	28
Vitality (% live)	59
Morphology (% normal)	3

2.4 Endocrine Disrupting Chemicals: Phthalates

Endocrine disrupting chemicals (EDCs) are a heterogeneous group of chemicals that mimic or interfere with the action of endogenous endocrine hormones. EDCs encompass hundreds of chemicals, either natural or manmade. Bisphenol A (BPA) and phthalates are 2 groups of widely used synthetic EDCs.

Phthalates, esters of phthalic acid, are used as plasticizers to impart flexibility, transparency and longevity to plastics. Phthalates were first commercialized as plasticizers in the 1920s. Phthalates are widely used in personal care products, cosmetics, air fresheners, soft toys, paint, lubricants, adhesives, medical equipment, flooring, and many other products (16, 172-175). It is estimated that 18 billion pounds of phthalates are manufactured throughout the world annually (17, 19). As phthalates are not covalently bound to polymers, they easily leach out of plastics into the environment (175, 176). Phthalates enter the food chain from air, water, soil, and food containers/packaging. Phthalates of potential health concern include: DBP, diisobutyl phthalate (DiBP), benzyl-butyl phthalate (BBP), butyl-benzyl phthalate (BBzP), di-*n*-pentyl phthalate (DnPP), DEHP, di-*n*-octyl phthalate (DnOP), diisononyl phthalate (DiNP) and diisodecyl phthalate (DIDP) (175). DEHP and DBP have been the most widely used phthalates. DEHP is commonly used in the polyvinyl chloride (PVC) market for its stability, fluidity and low volatility (177). Because of the widespread use of plastics, phthalate exposure is significant (15).

2.4.1 Physical and Chemical Properties

Phthalates are unique chemical compounds. Phthalates are alkyl/aryl esters of 1, 2-benzenedicarboxylic acid. The basic chemical structure of phthalates involves a benzene ring attached to two carboxylic acids at carbon 1 and 2 positions of the benzene ring. The carboxylic acids are attached with either a dialkyl or alkyl/aryl ester side chain. Branching patterns and length of the dialkyl or alkyl/aryl side chains determine the molecular weight (i.e., high vs. low) and physiochemical properties of different phthalates. The side chain also influences the chemo-dynamics in the environment, as well as metabolism, toxicity and excretion of phthalates from the body (15, 177). The physical and chemical properties of DEHP are shown in Table 2.4.1.

Table 2.4.1: Physical and chemical properties of DEHP (taken and modified from Agency for Toxic Substances and Disease Registry (ATSDR),Toxicological Profile for DEHP, 2002) (16)

Properties	**Value and Reference**
Molecular weight	390.7 (178)
Color	Colorless (179)
Physical state	Liquid (177)
Melting point	47^0C (177)
Boiling point	384^0C (178)
Solubility: Water Organic	 Vary between 1.1-1200 µg/L (177) and 41 µg/L(180) distilled water at 25 ^{0}C Solubilized in mineral oil and hexane (180)
Partition coefficients: Log Kow Log Koc	 7.50 (177) 4.9-6 (177)
Vapor pressure	1.0x10-7 mmHg at 25^0C (177)
Henry's law constant:	1.71x10-5 atm-m3/mole at 25^0C (177)
Autoignition temperature	735^0F ($390\ ^0$C) (180)
Flashpoint	384^0F ($196\ ^0$C) open cup (179)
Flammability limits	No data
Explosive limits	0.3% (lower limit) (179) No data (upper limit)
Conversion factor	1 ppm=15.94 mg/m^3 (181)

2.4.2 Human Exposure to Phthalates

Humans are widely exposed to phthalates in day to day life. As additives, phthalates are not chemically bound to plastic polymers. It is possible for phthalates to easily enter into the food chain because they leach from their matrix into the soil and water, and can be released

into the air (15, 16). Human exposure is possible via oral, inhalation, dermal and parenteral routes (15, 16, 17, 29, 175, 182, 183). Inhalation of phthalates in air particles and dust is a significant source of exposure (17, 184). Exposure to high molecular weight (HMW) phthalates, such as DEHP and DiNP, occurs mainly through the oral route. Soil has been a notable source of exposure to HMW phthalates for toddlers (184). Patients who require artificial ventilation, dialysis, intravenous fluids and critical medical care are also exposed to DEHP via the parenteral route. HMW phthalates are used to manufacture food packaging, building materials, and medical devices. Exposure to low molecular weight phthalates, such as DEP and BBP, occurs mainly through the skin (15, 17, 173). Low molecular weight (LMW) phthalates are used to produce personal care products (PCP), flooring, paints and adhesives. According to the US Environmental Protection Agency (EPA), the reference doses (RfD) of DEHP, BBP, DBP and DEP have been 20, 200, 100 and 800 μg/kg/day, respectively (17, 184). The daily intake of DEHP and DBP in the general population has been greater than other phthalates due to their widespread application (184).

Possible external sources of phthalate exposure and proportional contributions from different sources have been investigated. The summary of all potential sources of common phthalates are shown in Table 2.4.2. The contributions from all possible food and environmental sources in China has been reported to be the following: air (43–50%), drinking water (24–34%), foodstuffs (19–24.5%), dust (3–5.5%) and soil (<0.2%) (184). In the same study, the daily intakes (ng/kg body weight/day) of six phthalates were measured as: DBP (277–368), DEHP (149–203), DMP (dimethyl phthalate, 97–131), DEP (25–37), BBP (1.8–2.5), and DOP (1.5–2.1) (184). The estimated daily exposure to DEHP (excluding other types of exposure, such as occupational, medical and non-dietary ingestions in children) was from 3-30 μg/kg body weight (17, 185). Exposure to phthalates in children is higher than adults due to their frequent hand-to-oral contact (185). Children tend to be more susceptible to toxicity because of higher metabolic conversion to active metabolites and greater surface area to weight ratio compared to the adult (29, 185, 186). Occupational and medical exposures to DEHP (e.g., dialysis, respiration therapy, blood transfusions, or total parenteral nutrition treatment) are considerably greater compared to daily exposures in the general population (17, 183). Exposure has been estimated to be 700 μg /kg /day via inhalation in the plastics manufacturing workplace and 457 μg /kg /day for a patient undergoing dialysis or a blood transfusion (17, 183, 187). Phthalate metabolites have been

detected in the urine of American residents at levels above the exposure detection limit. It has been also reported that the number of PCP (e.g., soaps, body washes, shampoos, and cosmetics) used by adult women is associated with higher concentrations of certain urinary phthalate metabolites (172). After exposure via the different routes, phthalates undergo absorption, distribution, metabolism and excretion from the body.

Table 2.4.2: Summary of all possible sources of exposure to common phthalates and observed adverse outcomes in animals (modified from Hauser *et al.,* 2005) (15).

Phthalates	**Possible exposure sources**	**Commonly observed health effects (in animals, at high doses)**
DEP	Personal care products (e.g., fragrances), coatings, paintings, and pesticides	Reduced growth rate, reduced food consumption, increased organ weight
DnBP	Cellulose acetate plastics, personal care products, repellants, polishes, pharmaceutical coatings	Hepatic and renal effects, developmental and reproductive effects, decreased fetal weight
BBP	Vinyl flooring, adhesives and sealants, car care products, toys, food packaging, industrial solvents, PCPs (e.g., soaps fragrances, lotions, nail polishes)	Testicular toxicity, cryptorchidism, short ano-genital distance, altered steroid hormone levels, teratogenic
DEHP	PVC plastics for domestic purposes, such as toys, floor, tiles, furniture, and wall painting, foods packaging; blood storage bags and other medical devices	Hepatocellular carcinoma, testicular toxicity, anovulation, decreased fetal growth and teratogenesis at high doses

2.4.3 Toxicokinetics of Phthalates

2.4.3.1 Absorption and Distribution

Absorption and distribution of phthalates in mammals varies depending on the route of exposure. Besides gastrointestinal absorption, phthalates can be absorbed through skin and lungs in both humans and rodents. Absorption of phthalates is greater via the gut compared to the dermal route. Interspecies differences in phthalate absorption have been explored (188). Absorption of DEHP from the gut has been shown to be lower in both humans and non-human primates compared to rodents. This finding may be explained by knowledge that the bio-activation enzymes are less active in non-human primates compared to rodents. Absorption of phthalates via the gut may also vary depending on the exposure dose. At low doses, DEHP is absorbed as one of the following two metabolites: mono (2-ethylhexyl) phthalate (MEHP) or mono (2-ethyl-5hydroxyhexyl) phthalate (MEHHP). In contrast, at high doses, some parent DEHP can be absorbed (189). Unhydrolyzed DEHP can be metabolized via non-specific esterases present in the blood and/ or tissues (16). After oral administration, the estimated absorptions were as high as 25% in humans, >55% in rats, and <55% in non-human primates (190). The absorption of DEHP via the human skin is very low, around 1.1% of a 24-hour dermal application (16). After being absorbed, DEHP and its metabolites are distributed throughout the body via the blood. In blood, approximately 80% of DEHP is bound to lipoproteins and the rest to albumin (189). In rodents and monkeys given oral DEHP, the liver has been shown to contain the greatest concentration of DEHP and its metabolites (16). The absorption and distribution of oral phthalates have not been well-characterized in humans. The internal disposition of phthalates in the body has been shown to be falsely high (17). The inaccuracy of measurement may have occurred because of contamination of biological samples during laboratory processing (15, 16). Another factor that must be addressed during the assessment of phthalate absorption is the contribution that phthalate metabolism has upon the measures of absorbed phthalates.

2.4.3.2 Metabolism and Excretion

Phthalates are lipophilic, and hence undergo phase-1 and phase-II metabolic conversion in order to become water soluble. Water soluble metabolites are readily excreted from the body.

Phthalates have short biological half-lives, metabolize quickly and do not accumulate. Metabolites of phthalates are primarily excreted in the urine and feces (15, 191)

2.4.3.2.1 Phase-I Biotransformation

In phase-I metabolism, phthalates are converted to bioactive monoesters. Phase-I activation takes place in the gut, liver and blood. The phase-I reaction involves hydrolysis of one of the ester bonds by hydrolytic enzymes, such as lipase or esterase. The monoesters of common phthalates are shown in Table 2.4.3. Phase-I biotransformation is different for HMW and LMW phthalates. For example, relatively polar and low molecular weight phthalates, such as DEP, are primarily metabolized into their hydrolytic monoesters. In contrast, high molecular weight phthalates (e.g., DEHP and DIDP) undergo hydrolysis followed by further secondary oxidation of the side chain. The result of phase-I oxidation reactions is the production of metabolites that are relatively more hydrophilic (15, 191).

Table 2.4.3: The hydrolyzed phase-1 metabolites of four diester phthalates

Parent diesters	**Primary monoesters**
BBzP, n-butylbenzyl phthalate	MBzP, mono-benzyl phthalate
DEP, diethyl phthalate	MEP, mono-ethyl phthalate
DEHP, di-(2-ethylhexyl) phthalate	MEHP, mono-(2-ethylhexyl) phthalate
DBP, di-*n*-butyl phthalate	MBP, mono-*n*-butyl phthalate

DEHP undergoes phase-1 hydrolysis and oxidation. In phase-1 hydrolysis, DEHP converted to a bioactive metabolite, MEHP. In phase-I oxidation MEHP undergoes several oxidation reactions and forms 30 secondary oxidized metabolites. The main 4 secondary oxidized metabolites are: mono-(2-ethyl-5hydroxyhexyl) phthalate (MEHHP), mono-(2-ethyl-5oxohexyl) phthalate (MEOHP), mono-[2-ethyl-5carboxypentyl] phthalate (5cx-MEPP) and mono-[2-(carboxymethyl)hexyl] phthalate (2cx-MMHP) (Table 2.4.4) (15, 191, 192). It has been reported that 5cx-MEPP and 2cx-MMHP represent chronic exposure to DEHP because the elimination half-life is long (15, 192). In contrast, MEHHP and MEOHP represent short-term

exposure (15, 192). Phthalate monoesters and oxidative metabolites are excreted from the body directly without further metabolism. For example, low molecular phthalates, such as DEP, are removed after phase-I hydrolysis mainly as free MEP in the urine. Alternatively, excretion may occur after phase-II bio-transformation (15, 29, 189, 191-193).

Table 2.4.4: The two-step phase-1 bio-transformation of DEHP

Parent Diester	**Phase-1 hydrolyzed metabolites**	**Phase-1 oxidative metabolites**
DEHP	MEHP	MEHHP, MEOHP, 5cx-MEPP and 2cx-MMHP

2.4.3.2.2 Phase II Biotransformation and Excretion

Phase-II biotransformation increases the water solubility of phase-I phthalate metabolites. Phase-II conversion occurs mainly in the liver. The phase-II reaction involves addition of a glucuronic acid to the bioactive compound by enzymatic conjugation, such as glucuronidation. In human and non-human primates, DEHP metabolites (approximately 65%) are excreted predominantly through urine in the form of glucuronide conjugates (16). Other metabolites of DEHP include free MEHP, oxidized metabolites and phthalic acids. In rabbits, the unmetabolized/unabsorbed DEHP is excreted through feces. Like DEHP, other high molecular weight phthalates (such as DiNP, DIDP and DnOP), are excreted as conjugated metabolites in the urine (15, 194, 195). DBP is excreted via the urine in low concentrations as conjugated or unconjugated MnBP or various oxidation products of MnBP; and a small amount is excreted as free phthalic acid (174, 196). Approximately 24 hours are needed to eliminate more than 50% or oral DEHP and 5-7 days are needed to excrete 80% of the total dose (188, 190, 197). Most of absorbed DBP is excreted from the body within 24 hours, and virtually all is cleared within 48 hours (174).

The kinetics and fractional elimination of primary and secondary phthalate metabolites have also been investigated. After a single oral dose of deuterated DEHP and DINP, urinary elimination was reported in the following order of magnitude: MEHHP>5cx-

MEHP>MEOHP>MEHP (198). More than 90% of the metabolites were eliminated via urine in the first 24 hours and the remaining metabolites were excreted in the next 24 hours. The half-lives of the metabolites were approximately 4-8 hours (198). Stephen *et al*., (2012) quantified and compared phthalate diesters and metabolites between human serum, sweat and urine (48). Elimination of certain phthalate metabolites (particularly MEHP) occurred at a greater concentration in sweat than in urine. In several participants, detection of DEHP in sweat exceeded that in blood sera; differential levels supported the concept of tissue bioaccumulation of phthalates (48). As phthalates are lipophilic, they tend to accumulate in the adipose tissue. Further research is needed to measure and compare phthalate concentrations among blood serum, blood cells and adipose tissue in humans.

2.4.3.3 Biomarkers of Phthalate Exposure

A reliable biomarker is needed to quantify phthalate exposure in specific human body fluids. There have been different recommendations regarding the ideal biomarker. Very few studies have been conducted to quantify diester phthalates in human serum (45, 48, 49, 54). Quantification of diester phthalates is limited to study with special population undergoing medical interventions (e.g., dialysis, ventilation, intravenous resuscitation) (15, 17). In many human studies, phthalate metabolites, monoesters and/or oxidized metabolites have been quantified in biological fluids to estimate exposure (18, 46, 47, 182, 199). Urinary monoester metabolites of some commonly used phthalates, such as DEP, DBP and BBP have been recommended as good indicators of phthalate exposure in humans (40). Concentrations of thirteen hydrolyzed and oxidized phthalate metabolites were quantified in the serum, urine and seminal plasma of Danish men (47). In this study, it was proposed that serum MEP and mono-isobutyl phthalate (MiBP) could be used as biomarkers of exposure because of the correlation between urinary and serum concentrations of MEP and MiBP. Urinary excretions of metabolites in order of highest to lowest concentrations were: MEHP, MEP, MiBP, MnBP and MBzP. Several metabolites were detected in much lesser concentrations in serum compared to urine; only MEP in seminal plasma correlated with that in urine and blood (47).

Pregnancy is a unique situation during which the fetus may be exposed to phthalates. The amniotic fluid undergoes complete turnover every 3 hours; therefore, there is continuous exposure to the fetus via the amniotic fluid (46). During pregnancy and lactation, the assessment

of maternal urine metabolites may not accurately extrapolate to fetal and neonatal exposure and phthalate transmission into amniotic fluid and breast milk, respectively (45, 46). Phthalate metabolites in maternal serum are more likely to represent fetal exposure via placental blood transfer.

Research to determine whether phthalates concentrations in body fluids and tissues are associated with adverse health risks is complex. The most appropriate body fluid and tissue and the best analytical compound that will reflect exposure must be found. Quantification of oxidized metabolites and monoesters in both urine and serum has been recommended to improve the evaluation of exposure to phthalates (15, 199). Kato *et al.,* conducted a study in 127 human participants to assess the exposure to DEHP using MEHP and two oxidized metabolites (MEOHP and MEHHP) as biomarkers in both urine and blood serum (199). The concentrations of oxidized metabolites excreted in urine, primarily in the glucuronidated form, were 10 times higher compared to the levels of MEHP. Oxidized metabolites were detected in <50% serum samples (mainly in the conjugated form) at a lower concentration than that in urine. As phthalates have short half-lives, concentrations will vary from time-to-time depending on daily activities.

Sampling technique and storage have been evaluated as confounders in quantifying concentrations. The frequency of sampling of urine has been taken into consideration in some studies (15, 200). Although a single urine sample at a single point in time was moderately predictive of exposure of a subject over 3 months, sampling urine on multiple occasions was recommended to determine exposures over weeks or months (15, 200). Day-to-day and month-to-month variability in urinary phthalate concentrations were reported over a 3 month-period (200). However, in another study, urine phthalate metabolite concentrations did not differ between two consecutive sampling days (201). Metabolite concentrations in the fresh vs. stored urine samples were not different (202).

2.4.4 Potential Adverse Effects of EDCs on Reproduction

2.4.4.1 Animal Studies

2.4.4.1.1 Phthalates and Animal Reproduction

Many animal studies have been conducted over the past 50 years to clarify the effects of phthalates on the body systems. Research in animals, predominantly on rats, has provided evidence that phthalates have the potential to interfere with reproduction and development (23, 203-205). DEHP and its metabolite MEHP have been shown to exert ovarian toxicity in cycling female rats (27, 28, 206). Toxicodynamic effects mediated by MEHP included: lengthening estrous cycles, inhibiting ovulation, and reducing preovulatory follicle diameter by inhibiting granulosa cell proliferation and decreasing circulatory estradiol. Disruption of estradiol biosynthesis resulted in increased serum FSH and inhibition of LH surge. Absence of the LH surge prevented ovulation and thus lowered progesterone levels. Other phthalate metabolites, such as MEP, mono-butyl phthalate (MBP) and mono-methyl phthalate (MMP) were not reported to exert any adverse effects on female reproduction. However, effects have been reported with DBP and BBzP; implantation failure has been observed in the exposed pregnant and pseudo-pregnant rats (20, 21)

Mechanisms to explain how phthalates interrupt estradiol biosynthesis have been under investigation. In rodents, it has been proposed that MEHP interferes with steroidogenesis in 2 ways: 1) by suppressing FSH-stimulated cAMP production (207, 208); and 2) by indirectly activating the intra-nuclear PPARs and decreasing aromatase transcription in granulosa cells (208). PPARs act as transcription factors and regulate gene expression. The activation of intra-nuclear PPARs results in a number of molecular consequences including decreased aromatase messenger ribonucleic acid (mRNA) expression, increased 17β-hydroxysteroid dehydrogenase (17β-HSD-IV) transcription, and increased fatty acid binding protein (FABP) synthesis (208). FABP transports MEHP inside cells. Phthalates have been also implicated as a mechanism to alter hormonal regulation of the HPG axis. In a study in ewes, DEHP was found to alter the LH pulse frequency by disrupting the HPG axis instead of exerting a direct toxic effect on the ovary (209).

Phthalates have been found to cause testicular toxicity in animals. Peroxisome proliferation and subsequent alterations in cholesterol and fatty acid metabolism have been established as mechanisms of DEHP induced liver toxicity. However, a similar role for DEHP in eliciting testicular and developmental toxicity has not been found (210). Rather, alterations of gonocytes and reductions in Sertoli cells have been implicated in testicular toxicity in neonatal rats (211, 212). Even a comparatively low dose of MEHP disrupted Sertoli cell–gonocyte physical interactions and suppressed Sertoli cell proliferation (212). Sertoli cell number was reduced in rats exposed to DEHP during neonatal and adult life (213). MEHP caused early collapse of Sertoli cell vimentin filaments, which was suggested to induce germ cell apoptosis (214). However, fertility status was undisturbed in adults who had been exposed to phthalates in prenatal life (215).

Testicular Leydig cells may also be a target of phthalate toxicity following perinatal exposure. It has been documented that decreased testosterone synthesis by the Leydig cells leads to "androgen deprivation syndrome" or "testicular dysgenesis syndrome (TDS)". This syndrome includes developmental abnormalities of the epididymis, seminal vesicles and prostate as well as hypospadias, cryptorchidism, persistent nipples, lowered sperm count and reduced AGD (22-24, 204, 205, 216-218).

The mode of action of phthalates on fetal testicular Leydig cells has been widely studied in animals. Oral exposure to DEHP, BBP, or DBP during critical periods of intra-uterine life (gestation days 12-21) have been shown to elicit adverse effects in rats (22). Hydrolyzed monoesters readily cross the rat placenta and reach the fetus (219, 220). DBP was shown to interrupt the testosterone biosynthetic pathway by down regulating most of the genes for enzymes involved in Leydig cell androgen biosynthesis and signaling. The important enzymes for testosterone biosynthesis that were targeted were: scavenger receptor class B-1, SRB1 (receptor for the high-density lipoprotein); steroidogenic acute regulator protein (StAR); P450 side-chain cleavage enzyme, P450scc (enzyme involved in the conversion of cholesterol to pregnenolone); 3beta-hydroxysteroid dehydrogenase, 3β-HSD (enzyme involved in the conversion of pregnenolone to progesterone); CYP17 (enzyme involved in the conversion progesterone to androstenedione; and c-kit (receptor for the stem-cell factor) genes in rats (22, 221). Alterations in cholesterol transport genes and impairment of cholesterol transport and

metabolism have been suggested as a plausible mechanisms of testosterone biosynthesis disruption in DBP exposed rats (221). DEHP exposed fetal rats expressed inactivation of insulin-like factor 3 *(insI3)* gene, which may have been related to altered gubernaculum development, undescended testis and cryptorchidism (222). Other developmental abnormalities reported following DEHP exposure in pregnant rodents included reduced implantation rate, increased fetal resorption, and decreased fetal body weight (223). Additional mechanisms of endocrine disruption for phthalates have been proposed. A recent study has investigated the "critical mechanism of toxicity" (CMTA) of four main types of phthalates (DHEP, DEP, DBP and BBP) at different concentrations in developing fish embryos. Oxidative stress has been suggested as the CMTA for DEHP and DEP (11).

2.4.4.1.2 BPA and Animal Reproduction

Research has been conducted in animals over the past 15 years to determine associations between BPA and reproductive dysfunction. Bisphenol A has been implicated in the induction of various physiological and pathological changes in the female reproductive system. Examples include inhibition of folliculogenesis and ovulation, recurrent miscarriage, the development of ovarian cysts, oviductal lesions, endometrial hyperplasia, uterine polyps, cervical sarcoma, mammary adenocarcinoma, changes in brain structure and chemistry, and changes in adult behavior (27, 28, 206). In animal studies, prenatal exposure to BPA at environmentally relevant doses adversely affected embryonic development of the female reproductive tract and reproductive function in adult life (5, 30, 224-226). BPA has been also been shown to interfere with multiple stages of oogenesis in the developing ovary. In BPA exposed female fetal rats, abnormal meiosis, interference with the encapsulation of oocytes into follicles, and disruption of maturation of oocytes in the adult ovary were reported (227-230).

In addition to direct effects on follicles and oocytes, BPA has been shown to affect steroidogenesis. It has been suggested that BPA exerts its effects by interfering with estrogen hormone-receptor interactions (31). BPA can act as both an agonist and a competitive inhibitor/antagonist of endogenous estrogen receptors in rats. BPA binds to nuclear estrogen receptors with a lower affinity (i.e., 10,000 -100,000 fold weaker) than 17-beta estradiol (E2). However, the affinity of BPA for G-protein coupled transmembrane estrogen receptors is unknown (7, 231). It is plausible that BPA may stimulate altered cellular function, abnormal cell

proliferation and organ development, and endocrine disruption even at extremely low levels via the indirect G-protein pathway. Like an endocrine hormone, a non-linear dose response relationship exists between BPA concentration and the number of bound receptors and the maximal observable biological effects (232). BPA does not need to be present at high levels to have an effect upon the endocrine system (233). Exposure to BPA has been also proven to alter the expression of homeobox genes (HOX-10 & 11) and may lead to reproductive tract malformation in female neonatal mice (4, 234). BPA has also been reported to interfere with epigenetic mechanisms (i.e., hypomethylation of cytosine-guanine dinucleotide in DNA) leading to alterations in developmental programming in mice (235-238). BPA has found to cause down regulation of vaginal estrogen receptors in the prenatally exposed female offspring leading to an altered vaginal morphology in the post-pubertal mice (225). Furthermore, fetal BPA exposure at a dose higher than the lowest-observed-adverse-effect level (LOAEL) in Sprague-Dawley rats led to dysregulation of the HPG axis and caused anovulation, development of ovarian cysts and infertility (146).

Effects of xenoestrogens, such as BPA or diethylstilbestrol (DES) have been explored during embryogenesis in male mice. Exposure to a low dose of BPA or DES triggered a long AGD, an increase in prostate size and a decrease in epididymal weight (239). It has been suggested that prostate enlargement occurs because of enhanced androgen receptor binding. In contrast, a high dose of DES triggered a decrease in prostate weight, short AGD, and small fibrotic descended or undescended testes and sterility; decreasing androgen receptor binding was thought to be the cause (239, 240). Adverse effects on pregnancy included an increase in post-implantation pregnancy loss and a decrease in litter size. Low sperm count and hormonal imbalances have been reported in adult rats exposed to BPA prenatally (241).

Direct effects of BPA on pubertal and adult reproductive function have been investigated. BPA may disrupt male fertility by altering the testicular expression of Sertoli cell junctional protein (SCJP) in developing testes (241). Treatment of mice with BPA during puberty resulted in a significant drop of free testosterone and the development of multinucleated giant cells in seminiferous tubules of the testis (242). However, no gross alterations in spermatogenesis have been reported in adult rats following in-utero exposure to BPA (243). Bisphenol A can act as an antagonist of DHT (32-34). Modulation of multiple steps of the activation and function of

androgen receptors (AR) has been observed. Bisphenol A inhibited androgen receptor binding directly or altered the interaction of the androgen receptor with its co-activator (ASC1) (33). In addition, BPA has been found to modify androgen-induced transcriptional activity and androgen receptor nuclear translocation (33).

2.4.4.2 Human Studies

2.4.4.2.1 Phthalates and Human Reproduction

Scientific findings obtained to date on the potential adverse effects of phthalates on human reproductive health have been inconclusive. Of the limited studies conducted, few focus on the female reproductive system. Most studies lacked sensitivity and selectivity, and have been limited by considerable background contamination.

The relationships between phthalate metabolites and reproductive dysfunction in men have been investigated. Different parameters of male fertility, such as semen quality and concentrations of steroid hormones have been examined. Urine MBP and MBzP levels have been associated with poor semen parameters and decreased serum testosterone, FSH, and LH in men (2, 15, 244-246). In 269 men, correlations between urinary phthalate metabolites and abnormal semen parameters were reported (247). The findings included positive associations between urinary concentrations of MEHHP, MEHP and mono-iso nonyl phthalate (MiNP) and decreased sperm motility; MBP and abnormal CASA parameters, such as straight-line velocity (VSL), curvilinear velocity (VCL), and linearity (LIN); MEHP and decreased testosterone, FSH and LH production; MBP and an increased sperm DNA damage; and MBzP, MBP, MEHP and MEP and sperm aneuploidy. In another study, comparisons between CASA parameters and different urinary phthalate metabolites, such as MEP, MBzP, MBP, MEHP and mono-methyl phthalate (MMP) were examined (248). No associations between urinary MEP, MBzP or MEHP concentrations and CASA parameters were detected. In contrast, MBP positively correlated with abnormal sperm motility and low sperm concentration; MBzP with abnormal sperm motility; and MEP with high sperm DNA damage (244). Duty *et al.* also investigated correlations between urine phthalate metabolites and serum reproductive hormone levels (249). The inverse relationships between MBzP and FSH, and MEHP and testosterone were found; however, there was a positive correlation between MBP and inhibin-B. The link between 13

urinary phthalate metabolites and serum testosterone levels was explored in different age groups from the national health and nutrition examination survey (NHANES). An inverse relation was found only between DEHP metabolites and serum testosterone among men 40-60 years of age (38). More recently, anti-androgenic effects of DEHP metabolites have been documented (250). The negative correlations between serum DEHP metabolites and serum testosterone, sex hormone-binding globulin, semen volume, and total sperm count have been reported (250).

Associations between phthalates and female reproductive dysfunctions have been also reported in only a few human studies. Women with endometriosis had significantly greater plasma DEHP concentrations compared to the control group, and 92.6% had detectable DEHP and /or MEHP in the peritoneal fluid (49). The study groups identified a need to further evaluate toxicokinetics of phthalate metabolites as a means to bio-monitor and assess risks due to phthalate exposure. High concentrations of serum phthalate (e.g., DEP, DBP, DMP, and DEHP and its major metabolite MEHP) were observed in the majority of Puerto Rican girls with premature thelarche (54). In a separate study, however, no relationship was found between pubertal maturation and neonatal DEHP exposure (251).

Phthalate concentrations have been compared between fertile and infertile couples. Concentrations of five hydrolyzed metabolites (MEHP, MEHHP, MEP, MBP, MBzP) in spot urine samples were compared between 56 infertile Italian couples undergoing assisted reproduction techniques for the diagnosis of endometriosis, tubal factors, ovulatory disorders, unexplained and male factor infertility, and 56 fertile couples (18). There were greater urinary excretion of phthalate metabolites in infertile couples compared to fertile couples; however, no differences were detected between men and women (18).

Phthalates have been quantified in amniotic fluid and breast milk to assess fetal and neonatal exposure. A positive linear correlation between maternal urine and fetal amniotic fluid concentrations of phthalate metabolites has been shown (46). Oxidative metabolites of phthalates were detected in amniotic fluid for the first time in this study; however, concentrations were much lower compared to the maternal urinary levels. These findings provided rationale for further research to assess fetal metabolism and adverse fetal health effect of phthalates. No correlation between metabolites in urine and breast milk was found (46).

2.4.4.2.2 Bisphenol A and Human Reproduction

Evidence demonstrating negative influences of BPA on human reproduction has been seen. Adverse effects of BPA during IVF treatment have been shown to include poor ovarian response to stimulation drugs, a reduction of the number and quality of mature oocytes and fertilized oocytes, and decreased fertilization rates (252, 253). Lowered peak serum estradiol levels following gonadotropin stimulation (254); abnormal semen parameters such as low sperm count, abnormal morphology, decreased motility, and increased sperm DNA damage (37); poor embryo quality (255); and implantation failure (256) have been further associated with BPA exposure in infertility patients. Concentrations of BPA were positively correlated with serum total/free testosterone concentrations in women with PCOS (257). Similarly, serum BPA was greater in women with PCOS (both with and without obesity) compared to a control group (258). Women with PCOS also had higher serum dehydroepiandrosterone sulfate (DHEAS) and androgens compared the non-PCOS women (258). However, it is not yet clear whether BPA is a cause or consequence of PCOS. High serum BPA might also be related to insulin resistance in women with PCOS (259). Women with endometriosis exhibited greater serum and urine BPA concentrations when compared to women without endometriosis (39, 260). Inverse relationships between urinary BPA concentrations and free androgen index (FAI, a ratio of testosterone to sex hormone binding globulin), estradiol, estradiol:testosterone ratio, thyroid stimulating hormone and inhibin-B were reported. On the contrary, positive associations between serum FSH and the FSH:inhibin ratio have been found in the men undergoing infertility treatment (261). No correlation was found between unexplained infertility and urinary BPA in Chinese men undergoing assisted reproduction (262). Patients with complex endometrial hyperplasia and cancer showed lower serum BPA levels compared to those with simple endometrial hyperplasia (263). Exposure to BPA has also been positively associated with obesity (264-266).

Exposure to BPA in the workplace has been investigated. Male participants working in epoxy resin plants who had high urinary BPA levels exhibited sexual dysfunction and poor sperm quality (267-270). BPA has also been found to alter reproductive hormone concentration in many studies. BPA exposed workers had greater urinary BPA and lower FSH than the unexposed control populations (271). High urinary BPA was associated with high serum SHBG in both men and women (272, 273). Exposure to BPA (via urine) has also been associated with a

low serum free androgen index and FAI:LH ratio in men (273). However, in a separate study, urinary BPA concentration and serum testosterone were not correlated (272). High serum prolactin levels have been reported in women exposed to BPA in their workplace (274).

There have been contradictory findings about Bisphenol A and adverse pregnancy outcomes. In some studies, it has been suggested that serum BPA in women is positively associated with recurrent miscarriages (275, 276), low birth weight (277) and premature delivery (in a pilot study) (278). However, in another study, no relationship between BPA and birth weight was detected (279, 280). Prenatal occupational exposure to BPA was positively correlated with a low neonatal birth weight and a short anogenital distance in infant boys (281). In contrast, concentrations of BPA in umbilical cord blood were not greater in cryptorchid newborn boys compared with the unaffected boys (controls) (282). Similarly, BPA and phthalate metabolites in maternal urine were not found to be greater in newborn boys with cryptorchism and hypospadiasis compared to those without the anomalies (283).

2.5 Methods for Quantifying Exposure to Serum Phthalates

2.5.1 Liquid Chromatography coupled with Mass Spectrometry (LC-MS/MS)

Liquid chromatography and mass spectrometry (LC-MS/MS) is often used to measure a wide range of compounds, such as pharmaceuticals, environmental contaminants, foods and industrial materials. Liquid chromatography separates compounds based on the differential partitioning between the mobile and stationary phases (283). In LC, the liquid phase is a liquid and the stationary phase is a column. Columns are of different sizes, and contain various retardant chemicals to separate compounds based on their physiochemical properties. The column is either normal or reversed phase. In a normal phase column, hydrophilic chemicals (such as silica, aluminum resins and spherisorbs) are used to retard the hydrophilic compounds. In contrast, in reversed phase columns, hydrophobic chemicals (such as alkyl-phenyl, C_{18}) enhance the separation of lipophilic compounds including phthalates. Compounds are detected according to their properties by use of ultraviolet light, fluorescence or electric conductivity (283). LC analytical methods qualify and quantify a compound based on the unique retention time and peak absorption in the electromagnetic wave length, respectively (283, 284). LC provides a good resolution chromatogram for a single compound; however, it cannot accurately

quantify multiple unknown compounds eluted at the same retention time. Thus, coupling of LC with mass spectrometry (MS) provides a powerful technique for accurately separating and detecting a range of compounds (286).

Liquid chromatography coupled with MS has become a method of choice for phthalate detection due to its high sensitivity, selectivity and reproducibility. After separation of compounds using LC, MS detects analytes based on the mass to charge ratio (m/z) (284). The MS system measures gas phase ions while the LC measures ions in the liquid phase. It is therefore necessary to vaporize the liquid and to apply a charge to the analyte in order for it to be analyzed. The most common ionization methods include chemical ionization (CI), electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) (287). Each method ionizes and vaporizes samples differently. For example, APCI applies a charge at a corona discharge needle while APPI uses ultraviolet light to apply a charge (287). Thus, the method used determines which compounds will be detected and the limits of detection. Charged ions are then passed through the mass analyzers of MS (286).

In mass spectrometry, different types of mass analyzers (such as quadruple, magnetic sector, time of flight (TOF), electrostatic sector and ion cyclotron resonance) are used for separation and identification of ions (286). Quadrupole and magnetic sector mass analyzers use an electric or magnetic field to separate ions, respectively. By comparison, TOF separates ions by time needed to pass the tube depending on the kinetic energy and velocity of the ions. A single quadrupole MS is only able to separate precursor molecules based on their m/z. However, in the triple quadrupole MS, the first quadrupole receives the precursor/parent molecule. The second quadrupole stabilizes the ion and impacts it with an inert gas causing each precursor ion to fragment, a condition which is called collision induced disassociation (CID). The third quadrupole detects the fragment/daughter ions based on the m/z ratio (286, 287). Use of triple quadrupole enhances the reliability of compound identification by identifying both precursor and fragment ions. The quantity of specific ions that hit the detector is then converted to a signal. This signal is manipulated by software to display different peaks for specific ions.

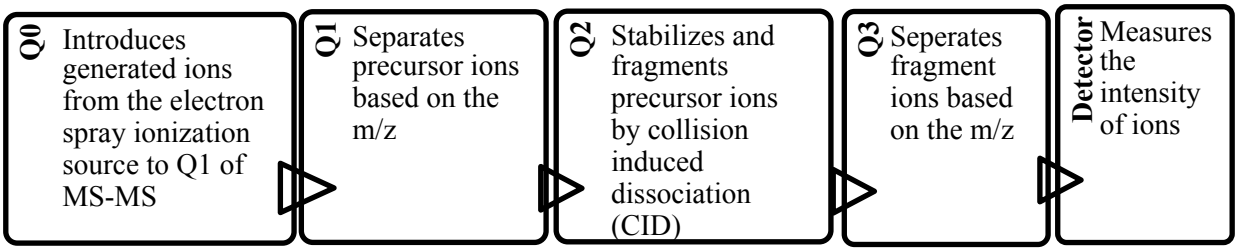

Figure 2.5.1: The triple quadrupoles of mass spectrometry (MS/MS). Specific functions of the triple quadrupoles are illustrated. The quadrupole Q1 detects precursor ions based on m/z; the quadrupole Q2 causes fragmentation of precursor ions by collision induced dissociation (CID); and the quadrupole Q3 detects product ions based on m/z. The m/z refers to mass to charge ratio.

2.5.2 Phthalate detection by use of Liquid versus Gas Chromatography coupled with MS

In addition to LC-MS, gas chromatography and mass spectrometry (GC-MS) has been used to quantify phthalate concentrations in biologic samples. There are some advantages of LC-MS over GC-MS. There is a wide range of acceptability of compounds that can be analyzed, including stable chemicals in a liquid or gaseous form, thermally unstable chemicals and least volatile compounds. There is less sample processing, no derivatization required, superior detection limit, and a shorter analysis time. There is a higher degree of confidence due to the presence of the quasi molecular ion and characteristic fragment ion (288). However, matrix interferences and background contamination from solvents and tubing are the two major problems associated with the use of LC-MS. By comparison, application of GC-MS is limited to only gases or volatile substances with high thermal stability. The GC-MS has lower limits of detection, and requires greater sample processing that in turn leads to greater contamination risk compared to LC-MS (288). Most analytical methods for phthalates from food matrices have utilized GC-MS (289). However, due to increasing reliability and sensitivity, LC-MS/MS methods are being developed. This method is often employed to study exposure, distribution and metabolic excretion of phthalates in various biological matrices (47, 290). Phthalates and metabolites have been detected in human urine, serum, breast milk, saliva, ovarian follicular fluid, seminal plasma and amniotic fluid samples by use of LC-MS/MS (41, 43, 290-295).

2.5.3 Phthalate extraction

Samples are purified and concentrated prior to phthalate quantification by LCMS. Both solid phase (SPE) and liquid/liquid (LLE) extraction methods have been employed and validated to extract phthalates. Extraction methods are applicable to cell cultures and plasma samples - fresh, frozen or archived. Phthalates have been extracted from milk and milk products (i.e., infant formula) using liquid/liquid extraction with a mixture of methyl *tert*-butyl ether (BME) and hexane (289). Milk fats were removed from DBP, BBP and DEHP by use of acetonitrile, while DINP and DIDP were isolated with use of deactivated silica. DEHP and MEHP have been simultaneously detected in seminal plasma by liquid-liquid extraction followed by isocratic reversed-phase chromatography with diode-array detection (295). Munch *et al*., used both LLE and SPE followed by GC-MS during the detection of phthalate esters in drinking water (296). In many human studies, phthalates metabolites were measured in biological matrices using automated SPE followed by LC-MS analysis (18, 44, 45, 48, 292, 293, 297). Silva *et al.*, quantified eleven phthalate metabolites in human urine using automated SPE (293). Both liquid and automated SPE were used before GC-MS analysis (45). The automated SPE method involved two extractions with hexane/methyl *tert*-butyl ether (1:1) and then one extraction with hexane, followed by separation on an amino propylene column. Enzymatic degradation of diester phthalates before extraction is a potential drawback as it can falsely depress concentrations of the parent compound in samples.

Hydrolytic enzymes present in biological fluids can hydrolyze diester phthalates. The hydrolysis of environmental diesters leads to falsely elevated concentration of monoesters in samples. In order to measure the enzymatic hydrolysis in samples, phthalate spiked serum samples were incubated at 37^0C with or without adding phosphoric acid (47, 199). Addition of phosphoric acid caused acid denaturation of the hydrolytic enzymes to eliminate potential enzymatic degradation of diester phthalates. Denaturation of enzymes by acid prevented false elevations or depressions of monoester and diester phthalates, respectively. In many studies, analytic methods have also incorporated enzymatic deconjugation of phthalate metabolites with β-glucuronidase (18, 45, 182). In order to measure conjugated metabolites, E. Coli K-12 β-glucuronidase enzyme and ammonium acetate buffer were added to samples and were incubated at 37 °C for 90 min (182, 293). However, no enzyme deconjugation was needed to measure free metabolites.

CHAPTER 3: RATIONALE

An increase in reproductive dysfunction, including infertility, has been observed in men and women over the past two decades. The current prevalence of infertility ranges from 11.5% to 15.7% in Canada (55), compared to a range of 3.5 to 26.4 % worldwide (298-300). More specifically, unexplained infertility is a common diagnosis among infertile couples, with a prevalence of 15%-30% (301). It has been postulated that phthalates are causally related to infertility in humans. This postulate is based on studies conducted primarily in animals (5, 22, 23, 203, 205, 208, 222). Few studies have been conducted to evaluate an association between EDC concentrations and human fertility. No studies have been conducted to quantify phthalates in men and women in Saskatchewan. Data from a proof of principle study in our lab revealed that there may be greater serum BPA concentration in male and/or female partners of couples diagnosed with unexplained infertility compared to fertile controls. We also found that phthalate concentrations in the cellular component of blood was greater in women with PCOS compared to controls (302), Baerwald *et al.*, unpublished data). Further investigations are needed to confirm our preliminary data.

In this study, blood, follicular fluid, and seminal plasma samples were collected from infertile couples undergoing IVF treatment. In addition, blood samples were collected from healthy fertile men and women with proven fertility. The original objective of our research was to quantify and compare concentrations of both phthalates and BPA in fertile versus infertile men and women. Due to unforeseen logistical limitations with study recruitment, equipment malfunctions, and attainment of research funding, we were only able to evaluate phthalate concentrations. Follicular fluid samples were evaluated. However, phthalate concentrations were undetectable using large sample aliquots. Seminal plasma samples have been collected but have not been analyzed.

CHAPTER 4: GENERAL OBJECTIVES

The main objective of the study contained in this thesis was:

> To determine whether serum phthalate concentrations differ in men and women with infertility compared to those without infertility in Saskatchewan.

CHAPTER 5: GENERAL HYPOTHESES

The hypotheses were that:

1. Serum phthalate concentrations would be greater in infertile versus fertile men.

2. Serum phthalate concentrations would be greater in infertile versus fertile women.

CHAPTER 6

Comparative Serum Phthalate Concentrations between Fertile versus Infertile Men and Women in Saskatchewan

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6.1 Abstract

Objective: To determine whether serum phthalate concentrations differ in men and women with infertility compared to those without infertility in Saskatchewan

Hypothesis: Serum phthalate concentrations will be greater in men and women with infertility compared to fertile men and women

Setting: Patients undergoing assisted reproduction for the treatment of infertility; healthy volunteers recruited from the community

Recruitment and sample collection: Infertile couples were recruited prior to in vitro fertilization (IVF) therapy for treatment of unexplained infertility (n=15), polycystic ovarian syndrome (PCOS, n=13), and male factor infertility (n=12). Fertile men (n=15) and women (n=15) were recruited using poster advertisements**.** Blood samples were collected by venipuncture for phthalate analysis.

Main outcome measures: Serum phthalates concentrations (ng/mL)

Design: Prospective cohort pilot study

Methods: In infertile couples, blood samples were collected on the following 3 days of the IVF cycle: early during ovarian stimulation, day of oocyte retrieval and day of embryo transfer. In healthy volunteers, 3 blood samples were collected over a 2 week period. Liquid chromatography and tandem mass spectrometry (LC-MS/MS) was conducted to quantify concentrations of four phthalates: di-*n*-butyl phthalate (DBP), diethyl phthalate (DEP), di-(2-ethylhexyl) phthalate (DEHP) and diisodecyl phthalate (DIDP). Phthalate concentrations were compared among the four study groups using non-parametric Kruskal-Wallis and Mann-Whitney U post hoc tests.

Results: Serum DEHP and DEP concentrations did not differ among control, unexplained, PCOS, and male factor infertility groups in both men and women ($p>0.05$). DBP in women did not differ among study groups ($p=0.205$). In contrast, DBP was lesser in men with unexplained, PCOS, and male factor infertility compared to controls ($p < 0.05$). Similarly, DIDP was lesser in women of couples with unexplained, PCOS and male factor infertility groups compared to fertile women ($p < 0.05$). Less DIDP was detected in men with unexplained and male factor infertility compared to the control group ($p < 0.05$)

Conclusion: Serum phthalate concentrations in serum were lesser or not different in infertility patients undergoing IVF compared to fertile volunteers. These findings do not support the notion that serum phthalate concentrations are associated with human infertility. Further research is needed to determine whether phthalate concentration in blood cells and adipose tissue differ in infertile versus fertile men and women.

Key words: Phthalates, PCOS, Unexplained infertility, Male factor infertility

Ms. Sandhya Roy participated in all aspects of conducting this study. She was involved in study design (20%) , participant recruitment (70 %), sample collection (90%), sample processing and LCMS analyses (90%), as well as data acquisition, statistical analyses and knowledge translation (90%).

6.2 Introduction

The human endocrine system is highly complex. The main functions of the endocrine system are to synthesize and release hormones which regulate the growth, development, and metabolism of organs and body systems. In addition to endogenous endocrine hormones, many chemicals in the environment can affect bodily processes. EDCs are hormonally active heterogeneous groups of exogenous agents. EDCs can mimic or interfere with the action of endogenous endocrine hormones including reproductive, thyroid, adrenal and pituitary hormones (1). Research conducted in animals, and to a lesser extent in humans, over the past 50 years has provided evidence that EDCs may cause a wide range of diseases and disabilities including obesity, diabetes, cancer, heart disease, thyroid disease, reproductive dysfunction, neurodevelopmental and neurodegenerative disorders (2). The most obvious effects of EDC are adverse effects on reproductive function and early fetal development (3-5).

Animal models have been used to investigate the biological effects of EDCs. Many animal studies have been conducted to understand the molecular mechanisms of action of EDCs, with a particular focus on toxicodynamic effects at low-dose exposures typical of environmental exposure (232, 233, 303). The EDCs may exert its effect(s) by a number of mechanisms including: 1) acting as an estrogen or aryl hydrocarbon receptor agonist, 2) acting as estrogen or androgen antagonist, 3) altering steroidogenic enzymes gene expression, 4) disrupting gonadotropin hormone production, 5) indirect interfering with steroidogenesis by alteration of cell membrane ion exchange, 6) inducing epigenetic changes, and/or 7) inducing oxidative stress (6-11). Direct effects on gametes (i.e., oocytes, sperm) have also been reported (12).

Hundreds of EDCs, either natural or man-made, are present in the environment. More than 500 chemicals are known or suspected to have endocrine disrupting potential (13, 14). Phthalates are a group of synthetic EDCs which are of particular concern because of their omnipresence, widespread use and high production (5). Women, men and children are continuously exposed to these chemicals through contaminants in the air, water, soil, dust, children's products, personal-care products (e.g., perfumes, lotions and cosmetics), construction materials, paint, medical devices and pharmaceuticals. Phthalates are a family of industrial compounds with a common chemical structure, dialkyl or alkyl/aryl esters of 1, 2-benzenedicarboxylic acid. Phthalates are primarily used to increase the flexibility, transparency,

durability and longevity of plastics (15, 16). As phthalates are not chemically bound to polymers, chemicals easily leach from their plastic matrix, become volatilized, move into the environment and ultimately enter into the food chain (15, 16). Worldwide, more than 8.2 million tons of phthalates are used every year. DEHP is the most commonly used, at 2 million tons of use per year (15-19).

Several adverse biological effects have been attributed to phthalates. Phthalates are not classified as carcinogens (International Agency for Research on Cancer). Rather, phthalates have been found to cause teratogenicity, developmental anomalies, and reproductive toxicity in animals (20-23). Phthalates, particularly DBP and DEHP have been studied to examine reproductive toxicity and developmental abnormalities in animals (23-26). Anti-androgenic effects, such as short anogenital distance (AGD), cryptorchidism, decreased testosterone concentrations and decreased sperm production have been reported after exposure in-utero (23-26). It has been also found that DEHP and its bioactive metabolite, MEHP cause ovarian toxicity in cycling rats by prolonging the estrous cycle, inhibiting ovulation and decreasing systemic estradiol (27, 28).

Most of the studies have been completed primarily in animal models; further research is needed to elucidate the impact of phthalates in humans. Metabolites of phthalates have been detected in human body fluids including urine, serum, breast milk, saliva, sweat, ovarian follicular fluid, seminal plasma, peritoneal fluid and amniotic fluid (3, 39-48). However, limited data, methodological inconsistencies, and high background contamination in many studies have led to contradictory and debatable results about potential adverse reproductive effects. Intrauterine exposure to low dose environmental phthalates has been associated with short AGD and incomplete testicular descent in men (35), similar to studies in animals. Inverse correlations between urinary phthalate metabolites, semen quality and reproductive hormones have been reported in men (15, 36-38). Phthalates have been termed as obesogens (29) due to their association with obesity. Low dose exposure, particularly during the fetal period, has been linked with childhood obesity (29). Associations between phthalate exposure and reproductive dysfunction have been explored in some studies (18, 39, 49). Greater phthalates and /or metabolites have been detected in women with endometriosis compared to the control group (49), and in infertile couples compared to fertile couples (18).

A decline in reproductive health has been observed in men and women within industrialized countries (51). The reasons for this decline are generally unknown. The worrisome decrease in fertility has been manifested as: reduced semen parameters, infertility and testicular cancer in men; various congenital abnormalities of the reproductive tract in both men and women; a decline in pregnancy and birthrate; and an early onset of puberty and menopause in women (50-53). Currently the prevalence of infertility in Canada (11.5-15.7%) is similar to that of other industrialized countries (10-15%) (55, 56). The underlying pathophysiology for many etiologies of infertility is not fully understood. Infertility is diagnosed when a couple fails to conceive within 12 months of unprotected intercourse. Unexplained infertility is diagnosed in 15%-30% of infertile couples and is characterized by no identifiable cause for reproductive dysfunction. Because of the associations reported between EDC exposure and reproductive dysfunction in animals, there has been concern that EDC may be causally related to human infertility. There is paucity of research to investigate the relationship between EDCs, particularly phthalates, and different types of human infertility. To date, no studies have been conducted to quantify BPA and phthalates concentration in men and women in Saskatchewan. Data from a pilot study in our lab have suggested that serum BPA concentration were greater in male and/or female partners of couples diagnosed with unexplained infertility compared to controls. We further found that phthalate concentration in the cellular component of blood were greater in women with PCOS compared to controls (Baerwald *et al*., unpublished data). Further research in a larger sample of men and women is needed to confirm these preliminary findings.

6.3 Materials and Methods

A prospective cohort pilot study was conducted in among the Department of Obstetrics, Gynecology and Reproductive Sciences at the University of Saskatchewan, the Toxicology Center at the University of Saskatchewan, and the Aurora Reproductive Care Center of Saskatoon. The study was completed from 2012 to 2014. Study procedures were conducted in accordance with the Tri-Council Policy Statement on the Ethical Conduct of Research Involving Humans. The study protocol was approved by the Biomedical Research Ethics Committee at the University of Saskatchewan (U of S) and the Strategic Planning and Priorities Committee at the Saskatoon Health Region (Appendix A). Informed consent was obtained from all participants prior to initiating study procedures (Appendix A).

6.3.1 Participant Recruitment:

Infertile couples undergoing assisted reproduction with the following diagnosis were recruited: unexplained infertility (n=15 male partners, n=15 female partners), PCOS (n=15 male partners, n=15 female partners) and male factor infertility (n=15 male partners, n=15 female partners), were designed to recruit. Infertile patients were enrolled from the Aurora Reproductive Care Center in Saskatoon, Saskatchewan. Both the male and female partners of infertile couples were required to participate. Couples with a combination of both male and female factor infertility were excluded.

Healthy volunteers (n=15 men, n=15 women) were enrolled. Inclusion criteria included proven fertility status and no history of reproductive dysfunction. Healthy volunteers were recruited from the University community and Saskatoon Health Region through electronic and paper advertisements.

6.3.2 Sample Collection and Preparation

Multiple blood samples were needed to determine the day to day variability of phthalate concentrations over time. Three samples were collected over a 2 week period in both fertile and infertile groups. Blood samples were collected from infertility patients on the following days during fertility treatment: (1) within the first 7 days of ovarian stimulation, (2) day of egg retrieval, and (3) day of embryo transfer. These 3 days were chosen based on the practicality of obtaining blood, follicular fluid and seminal plasma samples. Three blood samples were also collected from the healthy volunteers over a 2 week period. It was not possible to collect follicular fluid and seminal plasma from the healthy women and men, respectively.

At each visit, approximately 7 mL of blood was collected into a glass tube (BD Vacutainer #366340, 367342; Franklin Lakes, NJ USA). Blood samples were allowed to clot for 30 minutes, and then centrifuged at 2000 rpm for 20 minutes. The cellular and serum components of the blood samples were separated using glass pipettes and immediately frozen at -20°C in 15 mL polypropylene conical centrifuge tubes (Evergreen, Fisher Scientific, Cat#:08-558-33C) until analysis. Serum samples were transferred frozen to the Toxicology Center, and thawed at room temperature prior to extraction. All sample collection and preparation

procedures were conducted according to the Standard Operating Procedures (Environmental Toxicology Lab, University of Saskatchewan) (304-306).

6.3.3 Chemicals

All chemicals used in this study were HPLC grade or greater (Fisher Scientific, Ottawa, Ontario). As initial tests of solvents used for phthalate quantification indicated contamination, all chemicals were distilled prior to use and tested periodically.

6.3.4 Extraction

Thawed serum samples were mixed for 30 sec using a vortex shaker. Serum samples (0.5 mL) were transferred into 6 mL labeled glass culture tubes along with 10µl of deuterated internal standard, IS (500 ng/mL of D4-BBP) and 0.5 mL distilled methanol. After adding methanol, samples were again vortexed for 30 sec. Next, 2.5 mL of a 1:1 mixture of Hexane and MTBE was added to each tube. Each sample was vortexed for 1 min, and centrifuged at 2000 RPM for 2 minutes. The supernatant from each sample was carefully collected into a culture tube without disturbing the water fraction, and the extraction steps were repeated using Hexane and MTBE. The combined extract was evaporated under a gentle stream of nitrogen (N2). Samples were reconstituted with 4 mL 50/50 hexane acetonitrile (ACN) and vortex-mixed for 1 min. The top hexane layer was discarded and the bottom ACN fraction was collected and taken to dryness under steady nitrogen gas. Samples were reconstituted in 100 µL ACN and transferred to a gas chromatography vial (289, 307).

6.3.5 Instrumental Analysis

Phthalates in serum were quantified by LC-MS/MS using a HPLC (Agilent 1200 series Santa Clara, CA-95051) coupled to a PE Sciex triple quadruple mass spectrometer (API-3000 Framingham, USA). Separation of target analytes was performed using a Phenyl column (ACE Phenyl, 50 x 2.1mm, 3um Canadian Life Science Peterborough ON), with a sample run time of 18 mins. Solvents consisted of nanopure water with 0.1% acetic acid (solvent A), and distilled methanol with 0.1% acetic acid (solvent B). Initial operating conditions were 40% solvent A holding for 1 min changing to 100% solvent B over 2.5 min, and remaining at 100% solvent B for 13.5 minutes. A switch was made to 60% solvent B at 16 mins for the remaining 2 mins. A

guard column placed after the solvent pump was used to mitigate contamination from carrier solvents. The Mass Spectrometer, operating in positive mode ESI, used multiple reactions monitoring (MRM). Each phthalate compound was quantified using precursor and/or two product/daughter ions (Figure 6.3.1 and 6.3.2, Appendix B). The full list of compounds quantified is shown in Table 6.3.1. Mass spectrometer operating conditions were as follows: ion spray voltage=3500 volts, capillary temperature=350°C and gas flow rates=12, 8 and 12 AU for nebulizer, curtain and collision gases respectively. Concentrations were derived from a 12 point calibration curve of target analytes with a range from 1ng/mL to 0.01mg/mL. Concentrations > 0.01mg/mL caused depression of the internal standard, and did not distinguish the target peak from interference peaks generated from contamination. Samples were integrated using Analyst 4.1 software (Agilent Technologies).

Table 6.3.1: List of quantifying analytes including precursor and/or two quantifying fragment/daughter ions

Compound	Abbreviation	Molar Mass	Precursor Ions	Quantifying Ions (s)	
Diethyl phthalate	DEP	222.24	223	149	121
di-2-ethylhexyl phthalate	DEHP	390.56	391	149	167
Benzylbutyl phthalate	BBP	312.36	313	149	205
Diisodecyl phthalate	DIDP	446.66	447	149	205
Di-*n*-butyl phthalate	DBP	278.34	279	149	205
D4- benzylbutyl phthalate (IS)	D4-BBP	316.36	317	153	

6.3.6 Quality Assurance and Quality Control (QA/QC)

During all phases of this experiment, precautions were taken where possible to avoid phthalate contamination through the use of plastic materials. However, many items in the lab were potential unavoidable sources of contamination. Therefore, a wide range of blanks were generated to control these inputs. A schematic representation of background contamination and methods to assess and control for contamination in the reproductive and toxicology lab is shown in Figure 6.3.3.

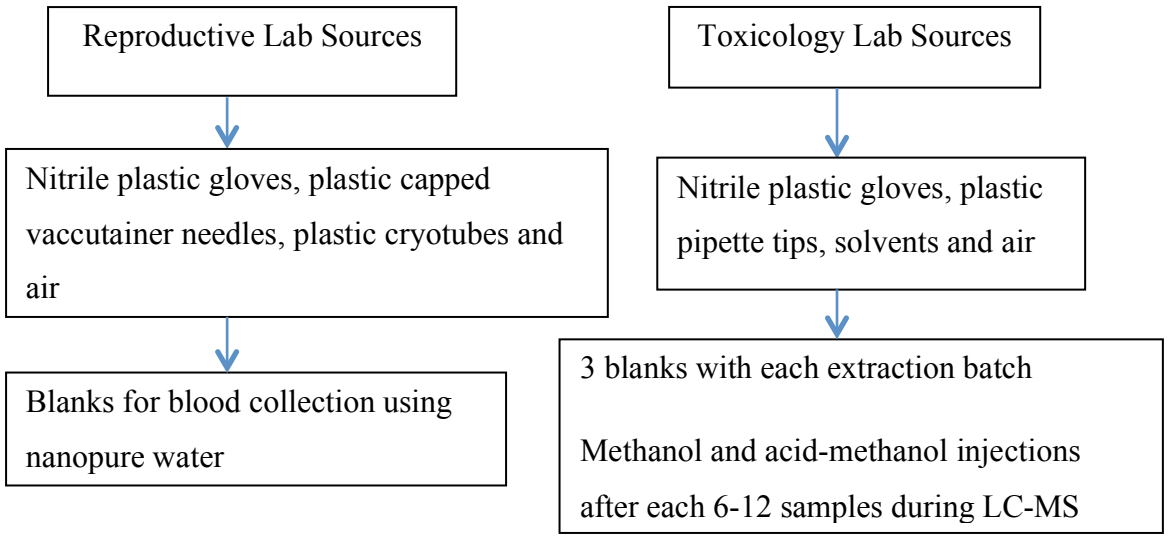


Figure 6.3.3: Potential sources of background contamination of phthalates and methods to assess and control for contamination from the reproductive and toxicology lab. On multiple occasions, nanopure water was exposed to all the glassware, plastics and chemicals that were used during sample collection, cryopreservation, extraction and analyses procedures to obtain an average background contamination for each phthalate.

For each set of 20 samples, there was a set of quality control samples that included a solvent blank, duplicate matrix blank (fetal bovine plasma for blood serum samples), and method blank (nanopure water). All samples were spiked with a 10 µL surrogate deuterated IS (D_4-BBP 500 ng/mL). The percent recovery of the IS (i.e., ratio of IS value in each sample versus average IS value in standards X 100) ranged from 12-50% with a median value of 29%. Matrix spikes were used to determine the accuracy of the extraction from blood serum and to monitor sample

matrix effects that could interfere with the analytes of interest. Surrogate spike samples were needed to monitor the quantitative transfer of the phthalate compounds throughout the sample extraction to LC-MS/MS detection phases. Method blanks included either a solvent or matrix blank. Blank samples included labware where nanopure water rather than plasma was added. Blanks were extracted along with the samples to monitor any contamination that might be introduced from sample collection, processing, storage and extraction.

Quality checks on the LC-MS/MS consisted of methanol and 1% acetic acid methanol injections every 6 and 12 samples respectively plus standard injections after 12 samples to check for instrument drift. Full 12 point calibration curves run prior to and after each batch of 40 samples were obtained by plotting peak area vs. concentration. Samples were integrated following a limit of detection (LOD) of 3 times the baseline. Methanol injection values were subtracted from sample values to account for contamination by the final solvent (which ranged from 0.3 to 4.6% of the mean detection of target analytes). Because initial sample runs indicated concentrations >0.01mg/mL for some analytes, samples were diluted 10 fold and reanalyzed to stay within the calibration curve. Standard calibration curves for HPLC analysis were formulated by plotting peak area and concentration. The equations for standard of DEHP, DEP, DBP and DIDP were obtained through regression analysis (Figure 6.3.4). The methanol subtracted sample value was divided by the corresponding IS value to calculate the sample response value ($\hat{y}$). Fixed concentrations (x) (ng/ml solvent/0.5 mL serum) for each phthalate were calculated by using value of $\hat{y}$, intercept (β_0) and slope (β_1) in the standard regression equation ($\hat{y}= \beta_1 x+\beta_0$). Final phthalate concentrations were obtained by dividing the x value by 10 (for ng/100 µL solvent), and then multiplying by 2 (for 1 mL serum) and 10 (for the dilution factor). Blood serum data were reported as ng/mL. The final phthalate concentrations in blanks and samples were evaluated to calculate the method detection limit (MDL) or LOD.

The nanopure water was exposed on multiple occasions to all the glassware, plastics and chemicals that were used during sample collection, cryopreservation, extraction and analyses procedures in order to obtain background contaminations. The average contamination for each phthalate of interest was calculated. The MDL was arbitrarily set at average of the background concentrations (3-fold value of the solvent blanks). Any sample value below this MDL was considered non-detectable. Phthalate concentrations which were above the MDL were

considered for further statistical analyses. We originally planned to quantify concentrations of five phthalates: DEHP, DEP, DBP, DiDP, and BBP. BBP was excluded from our analyses due to its detection below the MDL.

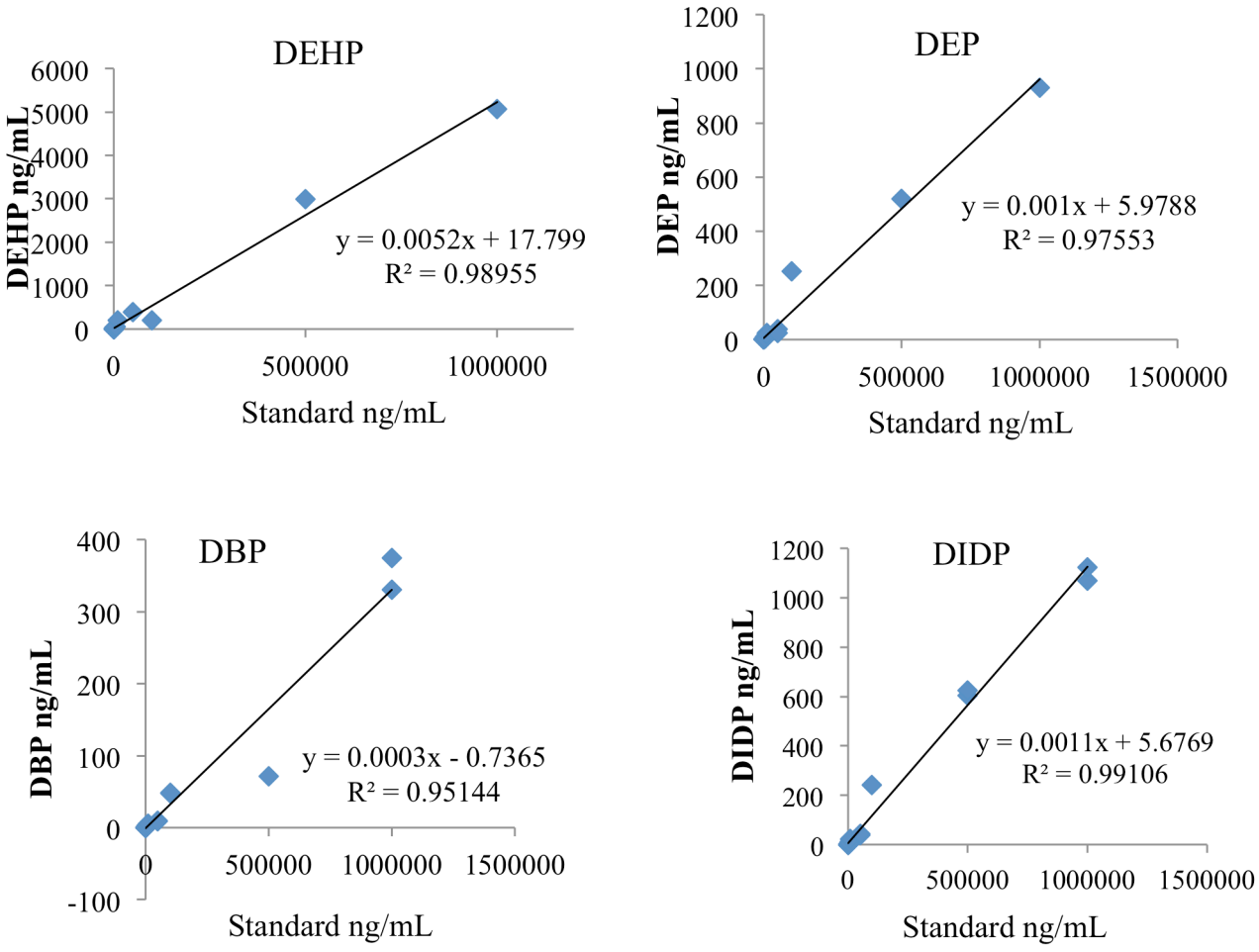


Figure 6.3.4: Standard curves showing the regression lines with regression equations and R^2 (regression coefficient) for DEHP, DEP, DBP and DIDP. Phthalates concentrations in samples were calculated by using the standard regression equations for each phthalate.

6.3.7 Statistical Analysis

Average concentrations of DEHP, DEP, DBP and DIDP over the 3 study visits were compared among the study groups using non-parametric Kruskal-Wallis and Mann-Whitney U post hoc tests (SPSS version 22). Only samples with values which were above the MDL were included in our analyses. Alpha was set at 0.05 for all statistical analyses.

6.4 Results

Average background contaminations from sample collection to toxicologic analysis procedures for different phthalates were quantified. The range of background phthalate concentration was approximately 500-26,000 ng/mL. The greatest contamination level was found for DBP and the least for DEP (Table 6.4.1).

Table 6.4.1: Mean background concentrations (ng/mL) of 4 phthalates with ranges intruded from the clinical and toxicologic analysis procedures.

Phthalates	**Background concentrations (ng/mL)**
DEHP	14,811 (11,026-17,713)
DEP	583 (513-689)
DBP	23,223 (21,216-26,228)
DIDP	13,720 (13,375-14,077)

Serum concentrations of DEHP were compared among control, unexplained, PCOS, and male factor infertility groups in men and women. No difference in serum DEHP concentrations were found among the 4 study groups for both men and women (Figure 6.4.1).

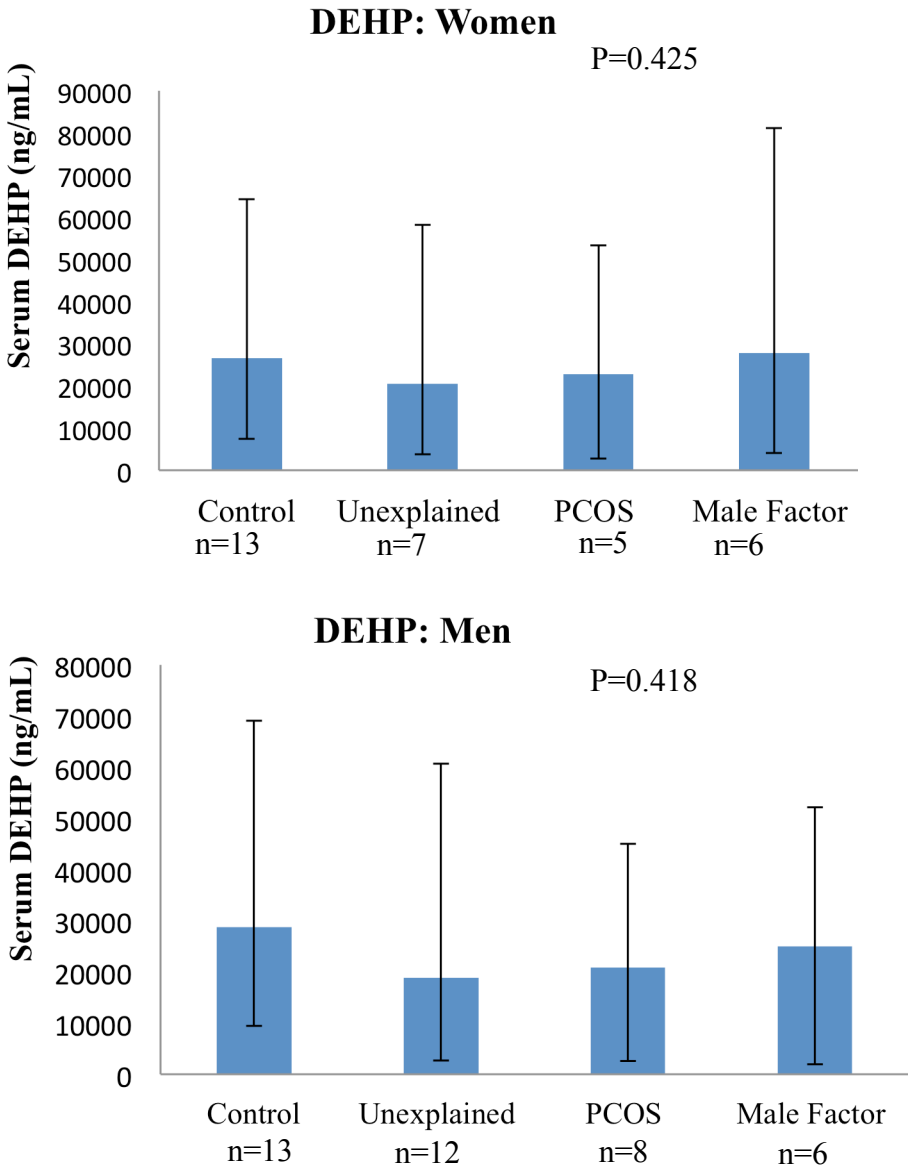


Figure 6.4.1: Comparisons of serum DEHP concentration among fertile versus infertile men and women. Data are shown as median ± interquartile range. The x-axis represents the 4 study groups and y-axis represents serum DEHP concentrations (ng/mL). Sample size in each group (data above MDL) is indicated by n.

Serum concentrations of DEP were compared among control, unexplained, PCOS, and male factor infertility in men and women. No differences in DEP concentrations were found among the 4 study groups for both men and women (Figure 6.4.2)

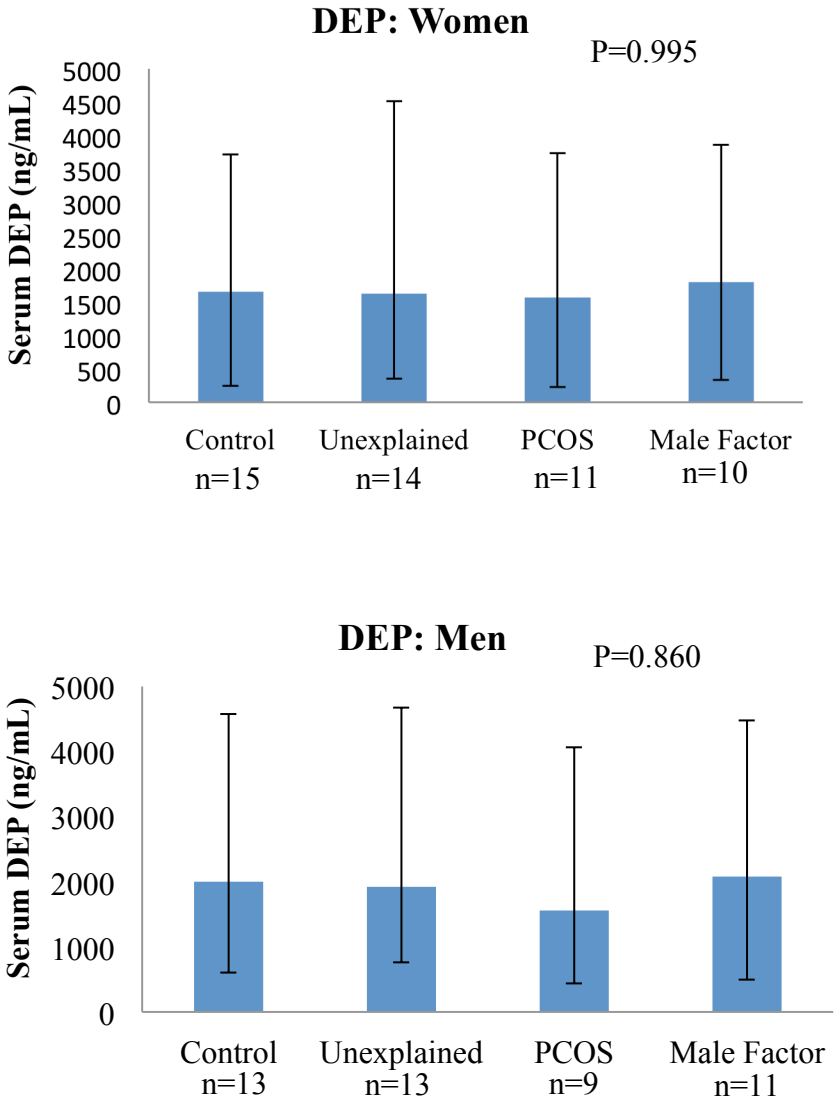


Figure 6.4.2: Comparisons of serum DEP concentration among fertile versus infertile men and women. Data are shown as median ± interquartile range. The x-axis represents the 4 study groups and the y-axis represents serum DEP concentrations (ng/mL). Sample size in each group (data above MDL) is indicated by n.

Serum concentrations of DBP in men and women were compared between control, unexplained, PCOS and male factor infertility groups. Serum DBP in women did not differ between the control, unexplained, PCOS and male factor infertility groups (p=0.205). However, mean serum DBP was less in men of couples with unexplained, PCOS and male factor infertility compared to the control group ($p < 0.05$) (Figure 6.4.3).

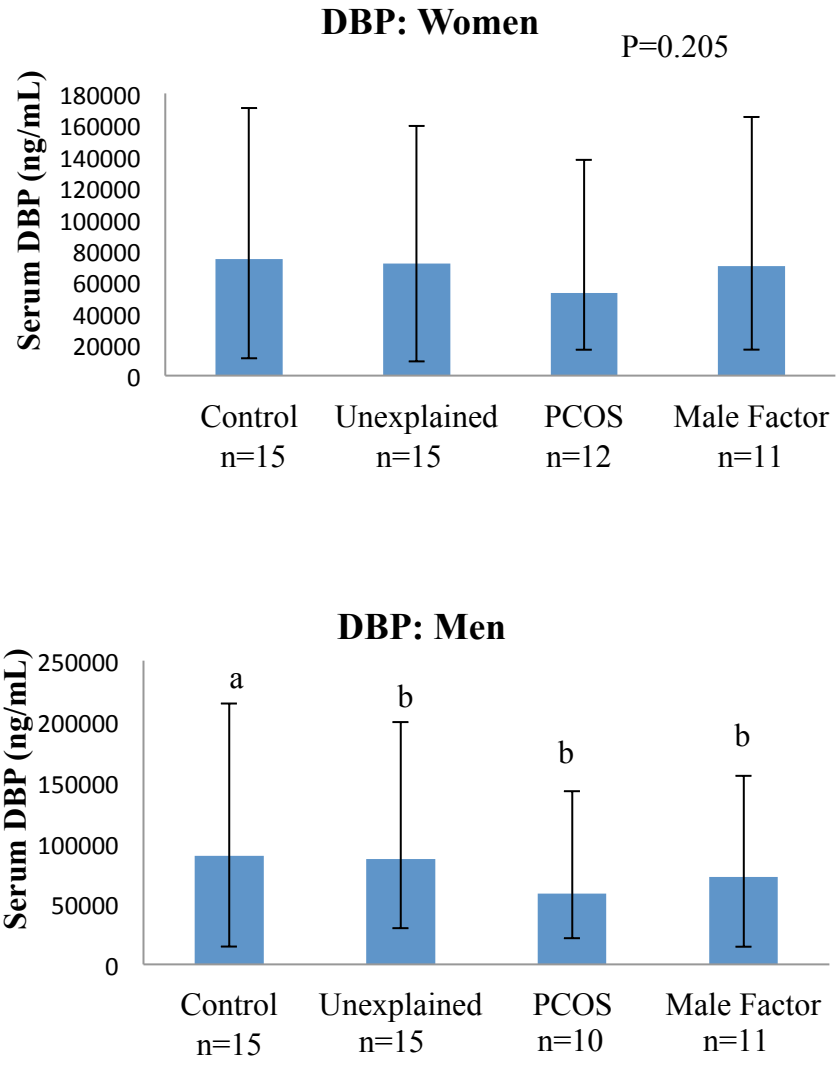


Figure 6.4.3: Comparisons of serum DBP concentration among fertile versus infertile men and women. Data are shown as median ± interquartile range. The x-axis represents the 4 study groups and the y-axis represents serum DBP concentrations (ng/mL). Sample size in each group (data above MDL) is indicated by n. [a,b] Groups with different superscripts were different ($p<0.05$).

Serum concentrations of DIDP in men and women were compared between control, unexplained, PCOS and male factor infertility groups. Serum DIDP in both women and men differed between the control, unexplained, PCOS, and male factor infertility ($p=0.015$ and $p=0.0005$). Serum DIDP in women were greater in the control group compared to unexplained, PCOS and male factor infertility groups ($p < 0.05$). Serum DIDP were greater in the control group compared to men of couples with unexplained and male factor infertility compared to controls ($p < 0.05$, Figure 6.4.4).

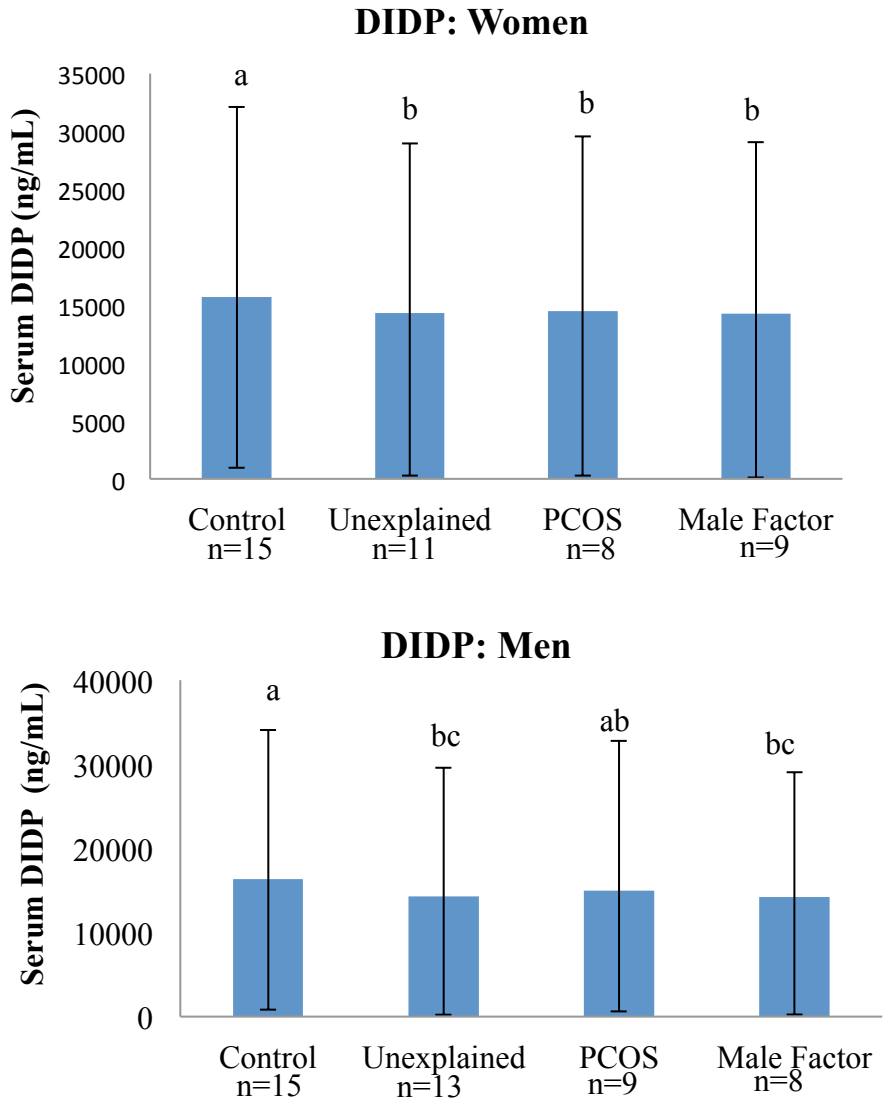


Figure 6.4.4: Comparisons of serum DIDP concentration among fertile versus infertile men and women. Data are shown as median ± interquartile range. The x-axis represents the 4 study groups and the y-axis represents serum DIDP concentrations (ng/mL). Sample size in each group (data above MDL) is indicated by n. [a,b,c] Groups with different superscripts were different ($p<0.05$).

A second set of analyses were conducted to regroup study participants. Specifically, male partners of women with PCOS and female partners of men with male factor infertility were included in the control groups. Serum DEHP was compared among control, unexplained and PCOS groups in women and control, unexplained and male factor infertility groups in men. No difference in serum DEHP concentrations were found among the 3 study groups for either men or women (Figure 6.4.5).

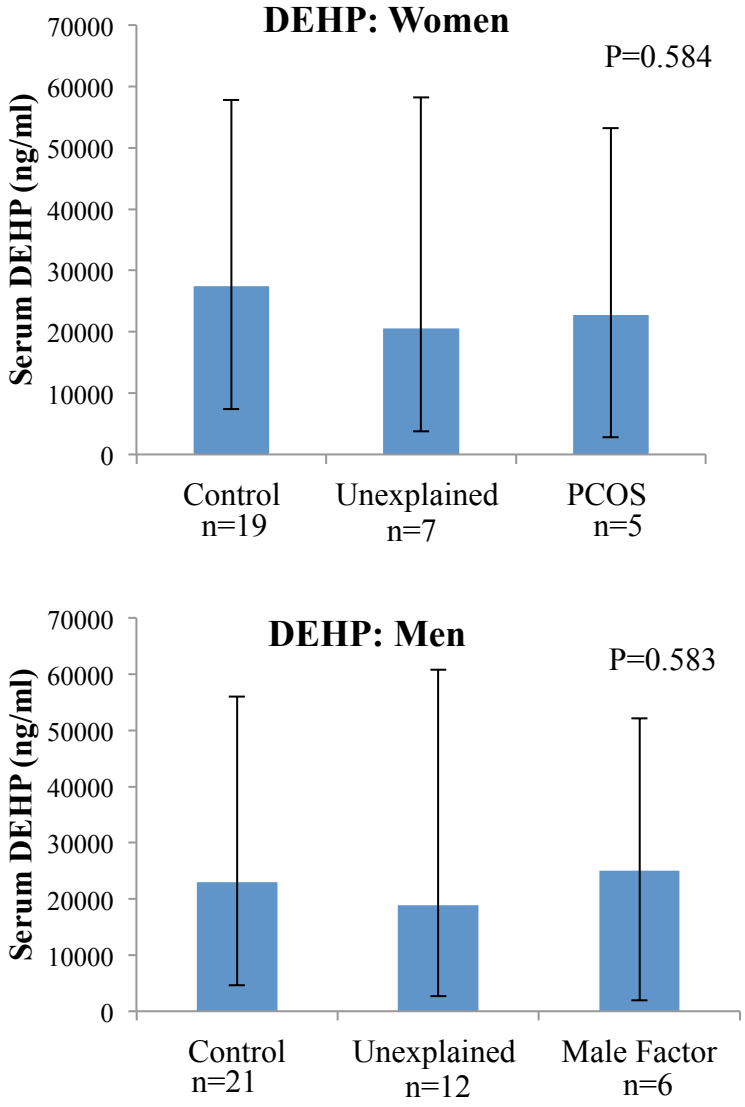


Figure 6.4.5: Results of secondary analyses conducted using modified study groups. Comparisons of serum DEHP concentration among fertile versus infertile men and women are reported. Data are shown as median ± interquartile range. The X-axis represents the 3 study groups and y-axis represents serum DEHP concentrations (ng/mL). Sample size in each group is indicated as n.

Serum DEP was compared among control, unexplained and PCOS groups in women and control, unexplained and male factor infertility groups in men. No differences in serum DEP concentrations were found among the 3 study groups for either men or women (Figure 6.4.6).

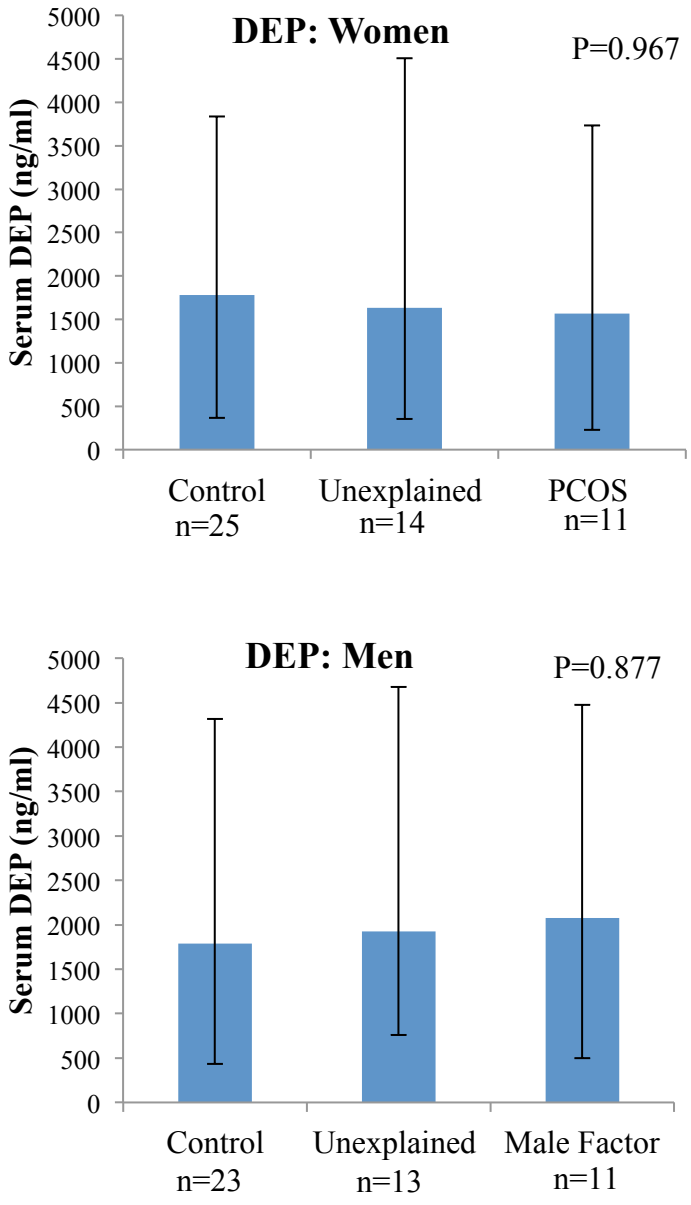


Figure 6.4.6: Results of secondary analyses conducted using modified study groups. Comparisons of serum DEP concentration among fertile versus infertile men and women are reported. Data are shown as median ± interquartile range. The X-axis represents the 3 study groups and y-axis represents serum DEP concentrations (ng/mL). Sample size in each group is indicated as n.

Serum DBP was compared among control, unexplained and PCOS groups in women and control, unexplained and male factor infertility groups in men. No difference in serum DBP concentrations were found among the 3 study groups for either men or women (Figure 6.4.7).

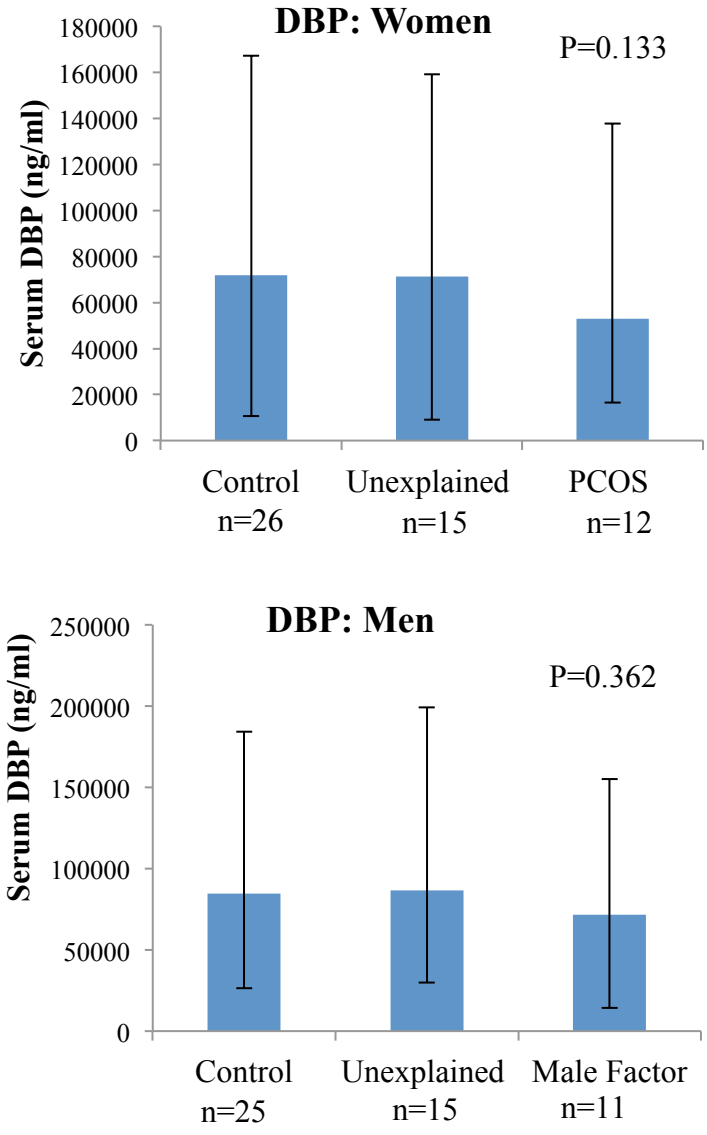


Figure 6.4.7: Results of secondary analyses conducted using modified study groups. Comparisons of serum DBP concentration among fertile versus infertile men and women are reported. Data are shown as median ± interquartile range. The X-axis represents the 3 study groups and y-axis represents serum DBP concentrations (ng/mL). Sample size in each group is indicated as n.

Serum DIDP were compared among control, unexplained and PCOS groups in women and control, unexplained and male factor infertility groups in men. No difference in serum DIDP concentrations were found among the 3 study groups for women. Serum DIDP was greater in the control group compared to unexplained and male factor infertility groups ($p < 0.05$) (Figure 6.4.8).

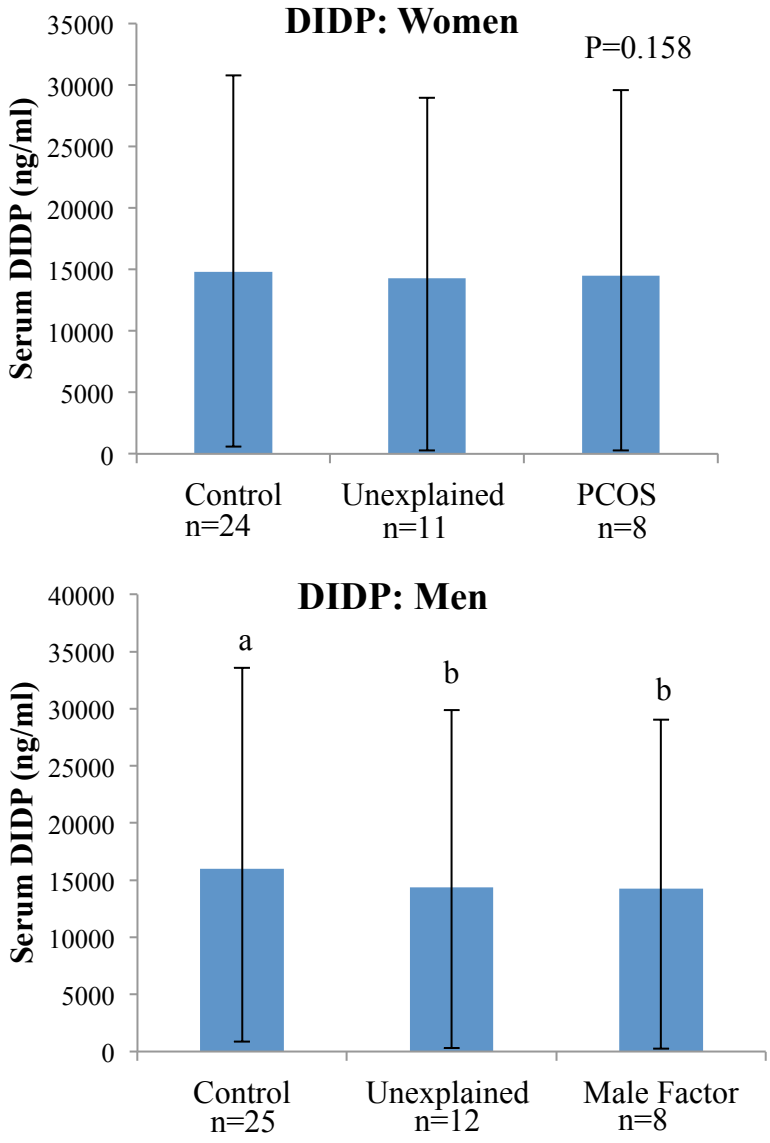
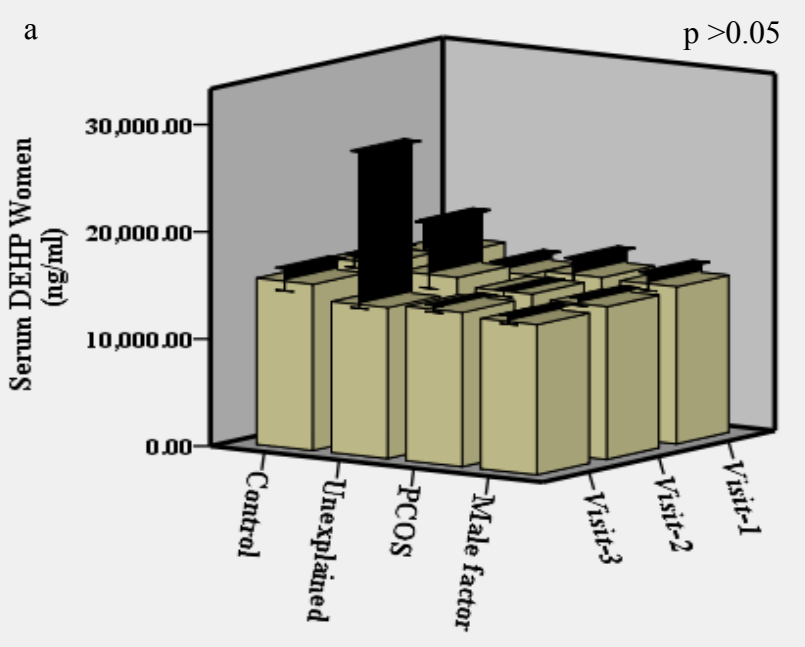
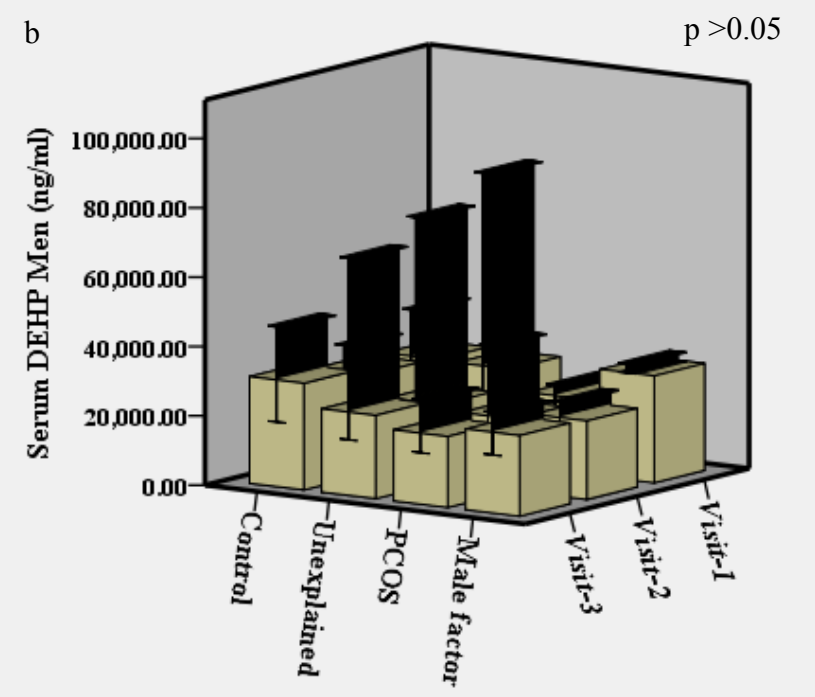


Figure 6.4.8: Results of secondary analyses conducted using modified study groups. Comparisons of serum DIDP concentration among fertile versus infertile men and women are reported. Data are shown as median ± interquartile range. The X-axis represents the 3 study groups and y-axis represents serum DIDP concentrations (ng/mL). Sample size in each group is indicated as n. [a,b] Groups with different superscripts were different ($p<0.05$).

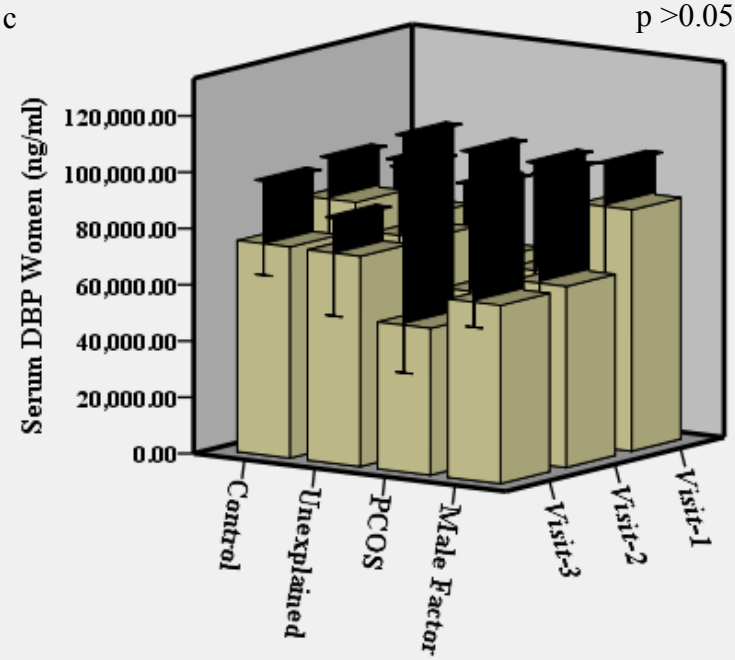
Serum concentrations of DEHP, DBP and DIDP were compared among the 3 study visits. No differences in serum DEHP, DBP and DIDP concentrations were observed among the 3 study visits for any of the study groups (Figure 6.4.9 a-f).





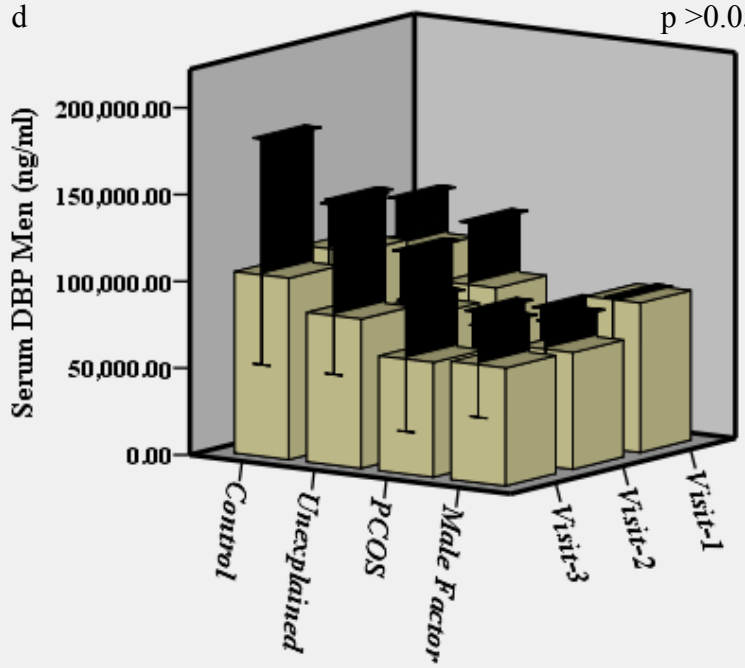
c

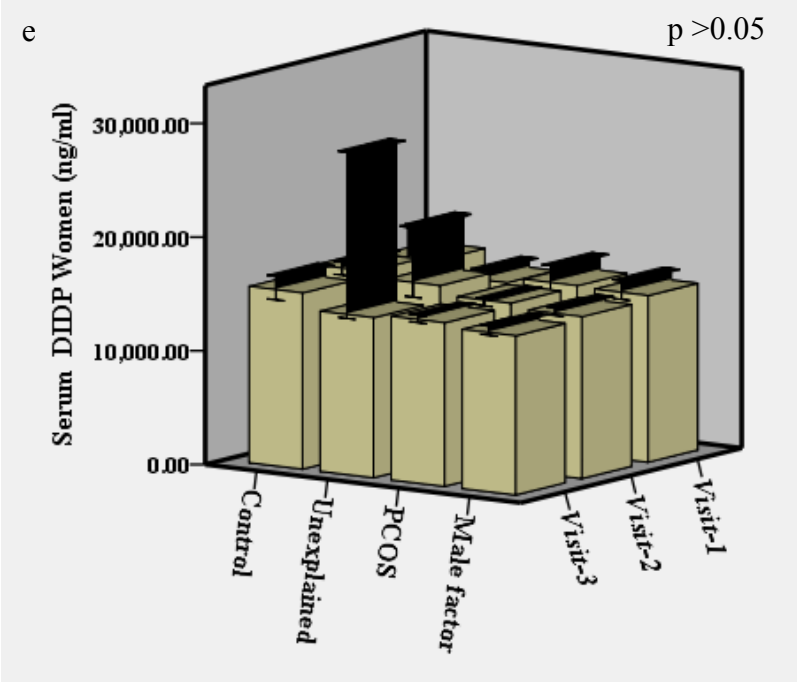
p >0.05


Serum DBP Women (ng/ml)
120,000.00
100,000.00
80,000.00
60,000.00
40,000.00
20,000.00
0.00
Control
Unexplained
PCOS
Male Factor
Visit-3
Visit-2
Visit-1

d

p >0.05


Serum DBP Men (ng/ml)
200,000.00
150,000.00
100,000.00
50,000.00
0.00
Control
Unexplained
PCOS
Male Factor
Visit-3
Visit-2
Visit-1



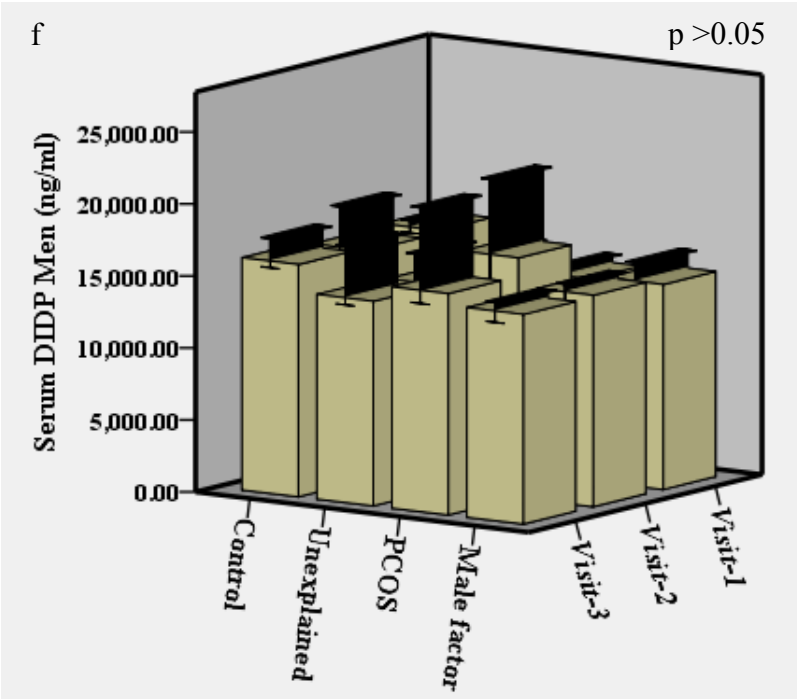


Figure 6.4.9: Comparisons of serum phthalate concentrations among the 3 visits in the 4 diagnostic groups (a-f). Data are shown as median $\pm$ standard error.

6.5 Discussion

In this pilot study, we quantified phthalate concentrations in human serum. After developing methods for quantification of phthalates (Giesy *et al.*, unpublished data), phthalate diester concentrations were compared among infertile and fertile men and women in Saskatchewan. Serum concentrations of DEHP and DEP were not different in infertile versus fertile men and women. On the contrary, in men, DBP was greater in the control group compared to infertility patients. Similarly, DIDP in both men and women were greater in the control group compared to infertility patients. Thus, our hypotheses were not supported.

Few studies have been conducted to elucidate potential adverse effects of phthalates on human fertility. Findings from our study do not support preliminary data previously obtained by our group and others. Since initiating our study, greater urinary phthalate metabolites have been reported in infertile versus fertile couples (18). In addition, serum DEHP levels have been found to be greater in women with versus without endometriosis (49). Inconsistencies among the present and previous studies may be attributed to differences in analytic compounds (metabolites vs. diesters) evaluated and body fluids (urine vs. serum) analyzed as well as variations in the sensitivity of analytical methods used.

In most human studies, phthalate metabolites have been quantified in urine samples. Very few studies have been conducted, to our knowledge, to quantify diester phthalates in human serum (45, 48, 49, 54). This is the first study in which human serum diester phthalates have been quantified in men and women of Saskatchewan. In the present study, the approximate median serum phthalate concentrations (ng/ml) in the fertile men and women were: DEHP: 29,000 and 26,500; DBP: 89,500 and 75,000; and DIDP: 16,500 and 15,500. Local Saskatchewan phthalate concentrations were remarkably greater (i.e., approximately 1000X) than those reported previously in Swedish postpartum (45). In comparison, median serum DEHP concentrations (μg/ml) in Italian women with endometriosis were reported to be approximately 100 times lower than those of infertility patients in our study (56). Similarly, median serum DEHP concentrations were 10 times lower in a previous study conducted in Puerto Rican pubertal girls (49) compared to the present study. Participant socio-demographic profiles, lifestyle factors (e.g., fresh vs. preserved foods), health status (e.g., healthy vs. hospitalized), pathophysiological conditions (e.g., endometriosis, premature thelarche), differences in methods used (LC-MS vs.

GC-MS) as well as assay sensitivity may in part, contribute to inconsistent findings among studies.

Several factors may have contributed to similar or lesser phthalate concentrations in infertility patients compared to fertile volunteers. First, in the process of obtaining consent, infertility patients were informed about emerging scientific evidence regarding EDCs and potential adverse reproductive effects. Knowing the financial and psychosocial burden of infertility treatment, couples may have subconsciously or consciously reduced phthalate exposures from all possible sources through lifestyle changes in order to optimize their chances of conceiving (although they were instructed not to do so). Second, BMI may be a potential confounder to our study findings. Since initiating our study, associations between BMI, lifestyle factors and phthalate exposure have been documented (308, 309). Third, infertile men and women may exhibit differential phthalate toxicokinetics (e.g., absorption, distribution, metabolism and excretion). It is possible that infertility patients may be genetically predisposed to metabolize phthalates more quickly, leading to lower concentrations compared to controls. Also, serum may not be the best endpoint to evaluate diester phthalates. The lipophilic nature of diester phthalates may result in sequestration in blood cells and fat, thereby leading to lower concentrations in serum. The notion of phthalate sequestration in tissues is supported by the finding that DEHP concentration in sweat exceeded levels found in urine or serum (48). In some human studies, environmental contaminants (i.e., p,p'-DDE, PCBs, BPA and heavy metals (cadmium, lead, mercury) were found to accumulate in follicular fluid in infertile women (310, 311). Phthalate concentrations in follicular fluid were evaluated in our study; however, no diester phthalates could be detected. Lastly, diesters may not be the best biomarker of phthalate exposure. The ubiquitous nature of phthalate diesters leads to ongoing challenges to reduce background contamination. It has been recently suggested that phthalate monoesters and/or their oxidized urinary metabolites may be a better biomarker than serum diester phthalates. Urinary oxidized metabolites were detected at greater concentrations (around 10 fold) and with lesser background contamination compared to the monoesters in urine (2, 15, 17, 47, 182).

Phthalates have short half-lives. Therefore, one may expect blood concentrations to vary from time-to-time, depending on daily activities. In this study, no differences in serum phthalate measurements were observed over the 2 week study period. We interpret these findings to mean

that phthalate exposures and thus serum levels do not vary substantially from day-to-day, due to their ubiquitous presence.

The biggest limitation of this study was the small sample size. With that said, the findings of this pilot study provide rationale for continued investigations to increase our understanding of potential effects of phthalates on the human reproduction. Another limitation to the study of phthalates in any lab is unavoidable background contamination. We made a conscious effort to minimize contamination by using phthalate-free labware and plastics with low levels of phthalates, whenever possible. With that said, potential sources of parent diester phthalate contamination included plastic blood storage tubes, plastic gloves, plastic pipette tips, solvents and air. Both LC-MS and GC-MS methods have been used for phthalate quantification. Both methods are continuously evolving and present both advantages and disadvantages. Since initiating our study, solid phase extraction followed by GC-MS has been proposed as an optimal method for measuring phthalate exposure, due to less background noise. However, this method is often cost-prohibitive, and involves more extensive sample processing.

6.6 Summary

In this pilot study, serum phthalate concentrations of DEHP, DEP, DBP and DIDP were either not different or less in men and women undergoing assisted reproduction compared to fertile controls. These findings are a first step in quantifying serum concentrations of phthalates in men and women of Saskatchewan. Our findings indicate widespread environmental phthalate exposures in both fertile and infertile populations, but at present, do not support the notion that phthalates have adverse effects on human fertility. Results of the present study provide rationale for continued research to quantify lipophilic diester phthalates in adipose tissue and blood cells, with additional consideration of BMI and lifestyle factors.

CHAPTER 7: GENERAL DISCUSSION

A prospective, observational pilot study was conducted among the Department of Obstetrics, Gynecology and Reproductive Science and the Toxicology Center at the University of Saskatchewan as well as AURORA Reproductive Care Center in Saskatoon, Saskatchewan. The objective of this collaborative project was to test the hypothesis that serum phthalate concentrations would be greater in infertile versus fertile men and women in Saskatchewan. Our hypothesis was based on data from a previous proof of principle study in which we reported the following: DEHP concentrations in the cellular component of blood were greater in women with PCOS compared to controls; and BPA concentrations in blood serum were greater in men and women with unexplained infertility compared to controls. In the present study, we found that phthalate concentrations were not greater in infertile patients compared to healthy fertile volunteers. Thus, the hypotheses in the present study were not supported.

There is a paucity of research to elucidate potential associations between human reproductive function and quantification of phthalate exposure. Information about phthalate concentrations in the environment and inside the human body is helpful to minimize potential health risks that may result from exposure. Investigations must be conducted in different geographic regions in order to determine region-specific potential adverse effects. This is the first study in which serum phthalate concentrations were evaluated in men and women in Saskatchewan. Median phthalate concentrations (ng/mL) in control groups were as follows: 1) DEHP: 28,703 in men and 26,534 in women, 2) DBP: 89,322 in men and 74,529 in women, 3) DIDP: 16,350 in men and 15,707 in women. The phthalate levels in our study were approximately 1000X greater those reported previously in Swedish postpartum women (45). Högberg *et al.* reported that the serum concentration of phthalates were ranged 0.31 to 5.9 ng/mL and their metabolites 0.77 to 1.8 ng/mL. However, the sensitivity of the assays in the Högberg's study was questioned providing a possible explanation for differences between studies. Similarly, median serum DEHP concentrations (µg/ml) were 10 times greater in the present study compared to a previous study in Puerto Rican girls between 6 months to 8 years of age with premature thelarche (56). The reported serum diester phthalate concentrations in the Peurto Rican girls were: DEHP (0.18- 2.0 µg/ml), DBP (0.01-0.27 µg/ml) and DEP (0.008-0.02 µg/ml). By comparison, serum phthalate concentrations in our study were: DEHP (26-28 µg/ml), DBP

(74-89 µg/ml) and DEP (0.6-5.8 µg/ml). Participant socio-demographic profiles, pathophysiological conditions, difference in methods used, lifestyle factors, health status, and sensitivity of analytical techniques may contribute to the discrepancies in phthalate concentrations between the studies.

Proportional contributions of phthalates from various environmental sources in different age groups have been reported in the following descending order: air (43–50%) > drinking water (24–34%) > foodstuffs (19–24.5%) > dust (3–5.5%) > soil (<0.2%). Moreover, the daily intake (ng/kg-body weight/day) of phthalates has been quantified in the following descending order: DBP (277–368) > DEHP (149–203) > DMP (97–131) > DEP (25–37) > BBP (1.8–2.5) > DOP (1.5–2.1) (184). After controlling for background contamination, we detected serum diester concentrations (µg/ml) in a similar pattern in the control groups: DBP (51-135), DEHP (15- 49) and DEP (0.6-5.8). DBP and then DEHP exceeded that of other phthalates in serum.

In a prior study, greater urinary metabolites have been reported in infertile versus fertile couples (18). Tranfo *et al.* (2012) studied Italian infertile couples undergoing treatment for endometriosis, unexplained infertility, tubal diseases, anovulation, and male infertility. Urinary metabolites (MEP, MEHP, MEHHP, MBzP and MnBP) were found to be greater in infertile versus fertile couples. By comparison, in our study we found that serum phthalates were either similar or greater in control groups compared to infertile groups. Although almost similar study groups and analytical methods were used between the 2 study, inconsistencies of findings may be attributed to differences in analytic compounds used (metabolites vs. diesters) and body fluids evaluated (urine vs. serum).

The unexpected findings of similar or lesser phthalate concentrations in infertility patients compared to fertile controls may be due to several factors. First, infertility patients were informed about emerging scientific evidence regarding EDCs and potential adverse reproductive health during the consenting process. Knowing the financial and psychosocial burden of infertility treatment, the couples may have subconsciously or consciously reduced phthalate exposures from all possible sources through lifestyle changes in order to optimize their chances of conceiving. Second, BMI may have confounded our study findings. Since initiating our study, associations between BMI and phthalate levels have been documented (308, 309). In one study, both external (daily intake) and internal exposure (metabolites in urine) to phthalates were

quantified in obese participants undergoing weight reduction compared to non-obese participants (308). No differences in urinary metabolite concentrations were observed between groups. However, urinary metabolites were elevated in obese participants that had lost weight over a 3 to 6 month period. In a separate study, positive correlations were detected between urinary phthalate metabolites and BMI as well as waist circumference (WC) in Chinese children (309). BMI data were not available for evaluation in the present study. Future studies in our laboratory and others should include BMI as a potential confounder for associations (or lack thereof) between phthalates and human fertility.

Third, infertile men and women may exhibit differential phthalate toxicokinetics compared to fertile controls. That is, absorption, distribution, metabolism, and excretion of phthalates may differ in infertile versus fertile people. For example, fertile couples might be genetically predisposed to metabolize phthalates more quickly, leading to lower concentrations compared to controls. In addition, blood serum may not be the best biological tissue sample to quantify diester phthalates. The lipophilic nature of diester phthalates may result in phthalates being sequestered in blood cells and fat, leading to lower concentrations in serum. The notion of phthalate sequestration in tissues is supported by the finding that DEHP concentrations in sweat exceeded that found in urine or serum (48). Lastly, serum phthalate diesters may not be the best endpoint to quantify internal phthalate exposures due to the ubiquitous nature of diesters. There is an ongoing challenge with high background contamination of research samples with ubiquitous environmental diesters.

Identifying an ideal biomarker for phthalate exposure in an appropriate body matrix has been a daunting task. In some studies, monoester phthalate metabolites in urine and or serum have been used as a biomarker of exposure (40, 47, 291). However, phthalate monoesters give a falsely elevated interpretation of exposure due to the contamination and breakdown of ubiquitous diester phthalates to their respective monoesters; this phenomenon of phthalate metabolism has been documented in many biological matrices, including urine, blood, seminal plasma, follicular fluid, amniotic fluid, peritoneal fluid and breast milk. Furthermore, formation of oxidative metabolites occurs following enzymatic breakdown within the body, rather than by hydrolytic conversion of background environmental diester phthalates. It has been suggested recently that oxidized metabolites in urine or serum may be a better biomarker to document exposure

compared to parent diester or monoester phthalates. Urinary oxidized phthalate metabolites have been detected at a greater concentration (around 10 fold) and with lesser background contamination, and were thus presumed to avoid falsely elevated exposure levels (15, 17, 47, 182). It is important to recognize that urinary metabolite concentrations may vary according to a participant's water intake and should be normalized by the urine creatinine value (18). Moreover, urinary maternal phthalates metabolites may not be the best predictor of fetal and newborn exposures via amniotic fluid and breast milk, respectively (45, 46). Determining the ideal biomarker(s) in appropriate body fluid or tissue to quantify internal phthalate distribution has been controversial and provides rationale for further research to elucidate phthalate quantification in adipose tissue versus blood cells and/or serum.

In some human studies, environmental contaminants (i.e., p,p'-DDE, PCBs, BPA, and heavy metals (cadmium, lead, mercury) were found to accumulate in FF in infertile women (310, 311). The results of these studies are contradictory (43, 312). In 5 women undergoing oocyte retrieval, phthalates and their metabolites were detected in FF at a concentration below15 ng/ml. This phthalate concentration in FF was much lower compared to that reported in animal ovarian studies (43). Similarly, BPA was not detected in labware used during the egg retrieval procedures in IVF (312). Collectively, these findings suggest that BPA and phthalates neither preferentially accumulated in FF nor leached out from the labware and media used in IVF treatment (43, 312). The main focus of the thesis work was to evaluate serum phthalate concentrations. However, blood cells, follicular fluid and seminal plasma samples were also collected. During our initial method development trial, we were not able to detect phthalates in a large volume of follicular fluid (i.e., up to 2 mL). Continued investigations are required to quantify phthalates in human blood cells, seminal plasma and adipose tissue.

Phthalates have a large octanol-water partition coefficient (i.e., Log Kow of DEHP = 7.5) which represents their high fat solubility. It is generally thought that due to rapid and efficient metabolism, phthalates do not accumulate in the body. However, findings of some studies have indicated the accumulation of phthalates in human tissues (48, 313, 314, 315). Phthalates have been classified as one of the lipophilic persistent organic pollutants (POPs) family which are resistant to biotransformation and excretion from the body (313). Other compounds belonging to this family are organochlorine pesticides, polychlorinated biphenyls (PCBs), dioxins, and

polybrominated flame retardants (BFRs) (313). POPs accumulate in white adipose tissue and cause potential disruption of nuclear transcription factors necessary for differentiation, metabolism and secretory functions of fat cells (313). Mes *et al.* (1974) quantified human postmortem DEHP and DBP concentrations at a range of 0.3-1 mg/ml and 0.1-0.3 mg/dl in adipose tissue. DBP and DEHP could also be detected in the fatty component of human kidney autopsies. These findings suggest a negative effect of phthalates on adipose tissue physiology and a potential relationship with pathological conditions such as diabetes, kidney disease, and PCOS. In some animal studies, evidence of phthalate tissue bioaccumulation has been provided (315, 316, 317). A significant proportion of phthalates from repeated small intravenous infusions was found to be retained for several months in the liver of rhesus monkeys (316). Similarly, in rats, the radioactivity of oral deuterated DEHP was reduced to only 1/3rd of the steady-state level in fat within 3 weeks (317).

Phthalates are ubiquitous and have short half-lives. As a result, it has been suggested that variations in serum concentrations may occur within a given day and across days. Quantifying phthalates in body fluids over weeks or months rather than at a single point in time has been recommended, due to the transient changes that may occur in phthalate concentrations following daily activities (15, 200). A single urine sample predicts phthalate exposure over 3 months; however, the predictabiliy varies among phthalate monoesters. For example, predicability is high for MEP but low for MEHP (200). In this study, three blood samples were collected to determine variations in concentrations over time. Serum concentrations did not differ among the 3 study visits over the 2 week period for any of the phthalates in any of the study groups. The lack of variability in serum phthalate levels observed over time supported previous research findings in which urine phthalate metabolite concentrations did not differ between two sampling days (201). Collectively, we interpreted these findings to mean that serum phthalate concentrations do not vary substantially from day-to-day due to their ubiquitous presence in the environment. However, in another study, day-to-day and month-to-month variability in phthalate metabolite concentrations in urine was found (200). The day-to-day variance of urine metabolites ranged from 27.2% (MBP) to 58.5% (MMP), and the cycle–to-cycle variance ranged from 1.5% (MBP) to 16.3% (MEP). Metabolite concentrations in the fresh vs. stored urine samples were compared and were not different (202). However, blood sampling frequency and effect of prolonged storage on blood parent phthalates and/or metabolites have not been studied.

Further research is warranted to evaluate fresh versus stored blood samples collected on multiple occasions over months to account for variability in exposure.

Due to the widespread presence of phthalates in the environment, background contamination from labware and equipment (e.g., solvents, reagents, glassware, air and others) is a fundamental problem when developing detection methods. With this in mind, special attention was made to reduce contamination in the present study. Whenever possible, approved glassware and specific plastics (e.g., use of nitrile plastic gloves, glass collection tube, vaccutainer needles versus collection sets with plastic tubing) were used to decrease contamination of samples. Blanks were run for several stages of study procedures to quantify background contamination. Blanks were run using nanopure water for sample collection, spinning, separation, cryopreservation, transportation, extraction and LC-MS analysis procedures. In addition, all chemicals used in this study were HPLC grade or better. All chemicals were distilled prior to use, and tested periodically. After minimizing all preventable sources, additional potential sources of parent diester phthalate contamination included plastic blood storage tubes, plastic gloves, plastic pipette tips, and solvents, as well as air in the labs. In future studies, minimizing background contamination through the use of dedicated and isolated laboratories with phthalate-free air and equipment would be ideal.

In the present study, male and female partners for each male or female infertility diagnostic group were evaluated separately. For example, female partners of couples with male factor infertility were considered as a separate female study group, rather than considering them as controls. Similarly, male partners of couples with PCOS were considered as a separate male study group rather than including them in the control group. This approach was used in order to prevent sampling bias. Because all couples were infertile, we could not be certain that the male partner of couples with PCOS or the female partner of couples with male factor infertility were truly fertile. In an effort to increase our sample size, we conducted a second set of analyses to expand our control groups. The male partners of women with PCOS and the female partners of men with male factor infertility were included in the control groups. After regrouping, the study groups were reduced to three instead of four: control, unexplained and PCOS infertility for women; and control, unexplained and male factor infertility for men. Secondary analyses of the modified study groups revealed that serum DIDP in men was greater in the control group

compared to unexplained and male factor groups. No other differences were observed. Thus, some of the differences in serum phthalates concentrations detected in our initial analyses were eliminated. When we considered the initial and secondary analyses collectively, we further concluded that serum phthalates were either not different or lesser in the infertile groups compared to the healthy fertile volunteers.

Limitations and future directions:

The results of our pilot study have been an important first step for developing methods to elucidate potential associations between serum phthalate levels and adverse reproductive health outcomes in Saskatchewan. The biggest limitation of this study was the small sample size. A sample size of n=15 per group was considered adequate for a pilot project, considering our proof of principle findings. Initially, the objectives of this research were to determine whether blood (serum and cells), follicular fluid and seminal plasma concentrations of BPA and phthalates differed in men and women with infertility compared to those without infertility. Due to the time constraints, limited funding, and unforeseen logistical challenges with study recruitment, we were not able to quantify BPA concentrations in serum samples. Furthermore, analyses of seminal plasma and blood cells have not been conducted. We anticipate continued evaluations of blood and seminal plasma samples collected.

A second limitation of our research was that the background phthalate contamination (i.e., values below the MDL) limited the final sample size for evaluation. As expected with relatively small datasets, exposure was not normally distributed. Therefore, non-parametric statistical evaluations were performed.

A third limitation of our study was that enzymatic degradation of diester phthalates by esterases in the serum may have led to falsely low quantification of diester phthalate. Hence, the inability to pretreat samples with acid to denature esterase enzymes during or after collection may be considered as a pit-fall of this study. With that said, freezing the samples shortly after they were obtained (within 60-90 min) would likely have minimized the enzymatic degradation of diester phthalates. In future studies, effort should be given to fix the enzymatic breakdown of diester phthalates by adding acid immediately after collection.

Additional research is required to optimize techniques to quantify phthalate concentrations using different body matrices. Comparisons of diester versus metabolites (monoesters and oxidized metabolites) phthalate concentrations are required. In order to improve methods, more specific knowledge about absorption, distribution, metabolism and excretion of phthalates through different routes of exposure is needed. We are particularly interested in quantification of diester phthalates by analysis of fat biopsies and the role of BMI on phthalate levels in the body, and potential associated negative reproductive health effects. Age, dietary habits, caloric intake, level of activity, travel, smoking, and disease conditions may be additional potential confounders to consider. The use of solid phase extraction (SPE) followed LC-MS or GC-MS should be considered when optimizing methods. Minimizing background contamination through the use of dedicated and isolated laboratories with phthalate-free air and equipment would be ideal.

In summary, serum phthalate concentrations of DEHP, DEP, DBP and DIDP were either similar or greater in fertile men and women compared to infertile men and women undergoing assisted reproduction treatment. Our findings do not support the notion that serum phthalates are associated with human infertility. The findings from this pilot study suggest a ubiquitous nature of phthalate exposure in fertile and infertile men and women of Saskatchewan. It is plausible that lifestyle factors and BMI may have confounded study findings. As phthalates are lipophilic, they may have a tendency to be sequestered into tissues, particularly in fat cells. Limitations aside, we believe that the results of our pilot study warrant continued investigations in a larger sample to document the potential effects of phthalates (in particular, in adipose tissue and blood cells) on human reproductive health.

CHAPTER 8: GENERAL CONCLUSION

The results of the pilot study included in this thesis have suggested the following:

1. Serum phthalate concentrations were not greater in infertile patients compared to healthy fertile volunteers:
    a) Median DBP concentrations were less in the male partners of couples with unexplained, PCOS, and male factor infertility compared to controls.
    b) Median DIDP concentrations were less in female partners of couples with unexplained, PCOS, and male factor infertility compared to controls.
    c) Median DIDP concentrations were less in male partners of couples with unexplained and male factor infertility compared to controls.

2. Phthalate concentrations in men and women within the study groups did not differ over the time

CHAPTER 9: REFERENCES

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APPENDIX A

Research Participant Information Sheet:
AURORA Patients

Endocrine Disrupting Chemicals: Potential Effects on Male and Female Reproductive Health in Saskatchewan

Principal Investigator: Angela Baerwald PhD[a]

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Study Sponsor: Royal University Hospital Foundation

Introduction:

You (the woman) and your partner (the man) are being invited to participate in a research study. Please read the following information sheet and ask as many questions as necessary before you decide whether to participate. Please take time to read the following information carefully and to discuss it with your family, friends, and doctor before you decide. Your participation in this study is entirely voluntary, so it is up to you to decide whether or not to take part. If you decide to take part in this study, you are both free to withdraw at any time without giving any reasons for your decision. If you choose to withdraw from this study, you will not lose the benefit of any medical care to which you are entitled or are presently receiving.

Who is Conducting the Study?

This study is being conducted by scientists and physicians in the Department of Obstetrics, Gynecology and Reproductive Sciences at the University of Saskatchewan and the Aurora Reproductive Care Center of Saskatoon. Funding to cover the administrative costs of conducting this study has been provided by the Royal University Hospital Foundation. The investigators are not being paid to conduct this study. The investigators have no known or potential conflicts of interest in conducting this study.

Why is this Study being Conducted?

Research performed primarily in animals over the past 50 years has shown that pollutants in the environment may be causing damage to the reproductive tissues, cells, and gametes (i.e., eggs and sperm). 'Endocrine Disrupting Chemicals' (EDCs) are of particular interest because they interfere with the production and/or action of hormones that control reproductive processes. Bisphenol A (BPA) and phthalates are EDCs to which Canadians are continuously exposed through food and beverage containers, air, water, soil, construction materials, and children's toys and products. BPA and phthalates have been detected in human serum, semen, amniotic fluid, follicular fluid (i.e., fluid in the sacs in the ovaries that contain the eggs), placental tissue, and breast milk; their metabolites have been measured in urine. However, the potential effects of exposure on human reproductive health are not fully understood. Furthermore, exposure to BPA and phthalates in people living in Saskatchewan has not been studied in detail.

The overall objective of this study is to determine whether environmental exposure to BPA and/or phthalates negatively affects reproductive function in women and men. We will determine whether concentrations of BPA and/or phthalates differ in men and women with different types of infertility diagnoses compared to men and women without infertility. Greater knowledge about the possible effects of BPA and phthalates on reproductive function may increase our understanding of the causes of infertility, assist in preventing reproductive disease, and improve patient care. We believe that the results of our study will be of interest to the general public and government agencies involved in regulating the manufacturing industry.

What does the Study Involve?

Couples undergoing IVF or ICSI for assisted reproduction as well as healthy male and female volunteers $\leq$ 40 years of age will be asked to participate. In order for infertility patients to be

eligible, both the male and female partners must agree to participate. Couples undergoing infertility treatment for the following reasons will be evaluated:

1. unexplained infertility (n=30 men, n=30 women)
2. PCOS (n=30 men, n=30 women)
3. Male factor infertility (n=30 men, n=30 women)

Couples with a combination of both male and female factor infertility will not be eligible to participate.

Additional participants will be assigned to the following control groups:

4. Healthy volunteers with no history or current diagnosis of reproductive dysfunction (n=30 men, n=30 women)

Pre-Study Visit
If you and your partner are eligible to participate in the study, you will be asked to visit Aurora Reproductive Care Center of Saskatoon for a pre-study appointment. At this appointment, study procedures will be discussed with you and you may ask any questions about the study. You and your partner will be asked to sign a consent form before initiating any study procedures.

Study Visits
If you agree to participate, you and your partner will undergo fertility treatment as per the normal standard of care. In other words, the fertility medications, ultrasound assessments, blood work during treatment, oocyte retrieval, semen collection, laboratory procedures and embryo transfer procedures that you undergo will not be affected by participating in this study.

i. Blood Samples:
You and your partner will be asked to provide 1 extra blood sample (approximately 7 mL or 1 teaspoon of blood) on each of the following regularly-scheduled appointments:

1. First ultrasound visit (approximately day 1 of FSH therapy)
2. Day of egg retrieval
3. Day of embryo transfer

Women normally undergo regular blood draws during their IVF treatment; therefore, one additional tube will be drawn for research purposes at the regularly-scheduled blood draw appointments. Men do not normally provide a blood sample during IVF treatment, but will be asked to do so in this study. Blood draws will be performed by phlebotomists (staff that specialize in drawing blood) physicians, nurses.

The blood samples will be processed to remove the cells, and the serum will be frozen for 6-9 months until the completion of the study.

ii. *Follicular Fluid Samples:*

The follicles in the woman's ovaries will be drained and the eggs will be retrieved from the follicular fluid, as per the standard of care. After the eggs are collected, the follicular fluid is normally thrown away. In this study, we will collect the fluid from the follicles on each ovary after the eggs are retrieved. The follicular fluid will be frozen for 12-24 months until study completion.

iii. *Seminal Plasma Samples:*

A semen sample will be provided by the man on the day of the oocyte retrieval procedure, as per standard of care. The semen sample is processed by allowing the semen to pass through a column to separate the most motile sperm (moving very quickly) from the less motile and non-motile sperm. The seminal plasma is the fluid portion of the semen that remains after the most motile sperm have been obtained. The seminal plasma is normally thrown away after the motile sperm are obtained. In this study, we will collect the seminal plasma and freeze it for 12-24 months until the study is completed.

At the completion of the study all of the frozen blood, follicular fluid, and seminal plasma samples will be transported to the Toxicology Center at the University of Saskatchewan. The levels of exposure to BPA and phthalates will be evaluated. The samples will be destroyed upon completion of the study.

You and your partner will have completed all study-related procedures once you undergo the embryo transfer procedures. The expected duration of study participation is approximately 13-20 days. The only procedure that differs from the normal standard of care are the 3 extra blood samples that are collected from each of the male and female participants, as well as the use of follicular fluid and seminal plasma that are normally thrown away.

What are the Possible Risks and Discomforts?

Potential risks during study participation include:

-discomfort and bruising at the site of the blood draw

What are the Benefits of Participating in this Study?

There are no direct benefits for you or your partner to participate in this study. We believe that the results of this study may increase our understanding of the causes of male and female infertility and assist in the development of better treatment options for couples experiencing infertility.

What Will the Study Cost Us?

You will not be charged for participating in this study. You will not be paid for participating in this study. You will be responsible to pay for your fertility treatment.

Will Our Participation be Kept Confidential?

One of the objectives of our study is compare IVF outcomes in patients with different levels of exposure to BPA and phthalates. Therefore, the results of your fertility treatment (eg. number and quality of follicles, oocytes and embryos that develop; fertilization rate, pregnancy rate) will be evaluated in this study. In Saskatchewan, the Health Information Protection Act (HIPA) protects

the privacy of you and your partner's personal health information. Your privacy will be respected. Your names will not be attached to any information, nor mentioned in any study report, nor be made available to anyone except the research team. It is the intention of the research team to publish results of this research in scientific journals and to present the findings at related conferences and workshops, but your identities will not be revealed.

What Happens if We Decide to Withdraw from the Study?

Your participation in this study is voluntary. You and/or your partner may decide not to participate or withdraw at any time. You and/or your partner do not have to provide a reason to withdraw from the study. If you choose not to participate in or withdraw from the study, it will not affect your future medical care.

Please notify the study personnel as soon as possible if either of you wish to withdraw from the study. If you voluntarily withdraw from the study and you do not want your data to be used in the study, your samples will be destroyed.

Who Do We Contact if We Have Questions About the Study?

If you or your partner have any questions regarding your participation in this study, please feel free to contact the Study Nurses (Jackie McVee, April Henry) at 306-653-5222 ext 239, Sandhya Roy (Research Co-ordinator) at 306-966-7851, Dr. Allison Case (Medical Investigator) at 306-653-5222, Dr. Adrian Gamelin (Medical Investigator) at 306-653-5222, Ms. Samantha Knott (Administrative Assistant) at 306-653-5222 or Dr. Angela Baerwald (Scientific Investigator) at 306-966-8073. You may also contact the study investigators once the study is completed to enquire about the results of the study.

If you have questions about your rights as a research subject or concerns about the study and your experiences while participating in this study, you can contact the Chair of the Biomedical Research Ethics Board at the University of Saskatchewan at 306-966-4053. The Research Ethics Board is a group of individuals (scientists, physicians, ethicists, lawyers and members of the community) that provide an independent review of human research studies. This study has been reviewed and approved on ethical grounds by the University of Saskatchewan Research Ethics Board.

Consent to Participate (Female Partner)

- o I have read (or someone has read to me) the information in this consent form.
- o I understand the purpose and procedures and the possible risks and benefits of the study.
- o I was given sufficient time to think about it.
- o I had the opportunity to ask questions and have received satisfactory answers.
- o I understand that I am free to withdraw from this study at any time for any reason and the decision to stop taking part will not affect my future medical care.
- o I give permission for the use and disclosure of my de-identified personal health information collected for the research purposes described in this form.
- o I understand that by signing this document I do not waive any of my legal rights.
- o I will be given a signed and dated copy of this consent form.

I agree to participate in this study:

Participant Signature: _____

Participant Name (please print): _____

Date: _____ Time: _____

Investigator/Delegate Signature: _____

Investigator/Delegate Name (please print): _____

Date: _____ Time: _____

Consent to Participate (Male Partner)

- o I have read (or someone has read to me) the information in this consent form.
- o I understand the purpose and procedures and the possible risks and benefits of the study.
- o I was given sufficient time to think about it.
- o I had the opportunity to ask questions and have received satisfactory answers.
- o I understand that I am free to withdraw from this study at any time for any reason and the decision to stop taking part will not affect my future medical care.
- o I give permission for the use and disclosure of my de-identified personal health information collected for the research purposes described in this form.
- o I understand that by signing this document I do not waive any of my legal rights.
- o I will be given a signed and dated copy of this consent form.

I agree to participate in this study:

Participant Signature: _____

Participant Name (please print): _____

Date: _____ Time: _____

Investigator/Delegate Signature: _____

Investigator/Delegate Name (please print): _____

Date: _____ Time: _____

Research Participant Information Sheet
Healthy Volunteers

Endocrine Disrupting Chemicals: Potential Effects on Male and Female Reproductive Health in Saskatchewan

Principal Investigator:	Angela Baerwald PhD[a]
Scientific Co-Investigators:	John Giesy PhD[a] Markus Hecker PhD[a] Paul Jones PhD[a]
Medical Investigators:	Donna Chizen MD FRCSC[a] Allison Case MD FRCSC[b] Adrian Gamelin MD FRCSC[b]
Study Nurses:	Jackie McVee BScN RN[b] April Henry RN[b]
Research Co-ordinator	Sandhya Roy MSc Candidate[a]
Study Location:	[a] Department of Obstetrics, Gynecology and Reproductive Sciences Royal University Hospital 103 Hospital Drive Saskatoon, SK S7N OW8 [b]AURORA Reproductive Care River Centre 1, 4th Floor 405-475 2nd Ave. S Saskatoon, SK S7K 1P4
Study Sponsor:	Royal University Hospital Foundation

Introduction:

You are being invited to participate in a research study at the University of Saskatchewan. Please read the following information sheet and ask as many questions as necessary before you decide whether to participate. Please take time to read the following information carefully and to discuss it with your family, friends, and doctor before you decide. Your participation in this study is entirely voluntary, so it is up to you to decide whether or not to take part. If you decide to take part in this study, you are free to withdraw at any time without giving any reasons for your decision. If you choose to withdraw from this study, you will not lose the benefit of any medical care to which you are entitled or are presently receiving.

Who is Conducting the Study?

This study is being conducted by scientists and physicians in the Department of Obstetrics, Gynecology and Reproductive Sciences at the University of Saskatchewan and the Aurora Reproductive Care Center. Funding to cover the administrative costs of conducting this study has been provided by the Royal University Hospital Foundation. The investigators are not being paid to conduct this study. The investigators have no known or potential conflicts of interest in conducting this study.

Why is this Study being Conducted?

Research performed primarily in animals over the past 50 years has shown that pollutants in the environment may be causing damage to the reproductive tissues, cells, and gametes (i.e., eggs and sperm). 'Endocrine Disrupting Chemicals' (EDCs) are of particular interest because they interfere with the production and/or action of hormones that control reproductive processes. Bisphenol A (BPA) and phthalates are EDCs to which Canadians are continuously exposed through food and beverage containers, air, water, soil, construction materials, and children's toys and products. BPA and phthalates have been detected in human serum, semen, amniotic fluid, follicular fluid (i.e., fluid in the sacs in the ovaries that contain the eggs), placental tissue, and breast milk; their metabolites have been measured in urine. However, the potential effects of exposure on human reproductive health are not fully understood. Furthermore, exposure to BPA and phthalates in people living in Saskatchewan has not been studied in detail.

The overall objective of this study is to determine whether environmental exposure to BPA and/or phthalates negatively affects reproductive function in women and men. We will determine whether concentrations of BPA and/or phthalates differ in men and women with different types of infertility diagnoses compared to men and women without infertility. Greater knowledge about the possible effects of BPA and phthalates on reproductive function may increase our understanding of the causes of infertility, assist in preventing reproductive disease, and improve patient care. We believe that the results of our study will be of interest to the general public and government agencies involved in regulating the manufacturing industry.

What does the Study Involve?

Couples undergoing IVF or ICSI for assisted reproduction as well as healthy male and female volunteers $\leq$ 40 years of age will be invited to participate. In order for infertility patients to be

eligible, both the male and female partners must agree to participate. Couples undergoing infertility treatment for the following reasons will be evaluated:

1. unexplained infertility (n=30 men, n=30 women)
2. PCOS (n=30 men, n=30 women)
3. Male factor infertility (n=30 men, n=30 women)

Couples with a combination of both male and female factor infertility will not be eligible to participate.

Additional participants will be assigned to the following control groups:

4. Healthy volunteers with no history or current diagnosis of reproductive dysfunction (n=30 men, n=30 women)

Pre-Study Visit
If you are eligible to participate in the study, you will be asked to visit the Royal University Hospital for a pre-study appointment. At this appointment, study procedures will be discussed with you and you may ask any questions about the study. You will be asked to sign a consent form before initiating any study procedures.

Study Visits
If you agree to participate, you will will be asked to provide 1 blood sample (approximately 7 mL or 1 teaspoon of blood) on 3 separate days. Blood draws will be performed by physicians, nurses and/or researchers with training in phlebotomy (i.e., drawing blood). Appointments for blood draws will be made approximately 4-7 days apart.

At the completion of the study all of the frozen blood samples will be transported to the Toxicology Center at the University of Saskatchewan. The levels of exposure to BPA and phthalates will be evaluated. The samples will be destroyed upon completion of the study.

You will have completed all study-related procedures once you undergo the 3 blood draws.

What are the Possible Risks and Discomforts?
Potential risks during study participation include:
-discomfort and bruising at the site of the blood draw

What are the Benefits of Participating in this Study?
There are no direct benefits for you or your partner to participate in this study. We believe that the results of this study may increase our understanding of the causes of male and female infertility and assist in the development of better treatment options for women with reproductive dysfunction.

What Will the Study Cost Me?

You will not be charged for participating in this study. You will not be paid for participating in this study.

Will My Participation be Kept Confidential?

In Saskatchewan, the Health Information Protection Act (HIPA) protects the privacy of your personal health information. Your privacy will be respected. Your name will not be attached to any information, nor mentioned in any study report, nor be made available to anyone except the research team. It is the intention of the research team to publish results of this research in scientific journals and to present the findings at related conferences and workshops, but your identity will not be revealed.

What Happens if I Decide to Withdraw from the Study?

Your participation in this study is voluntary. You may decide not to participate or withdraw at any time. You do not have to provide a reason to withdraw from the study. If you choose not to participate in or withdraw from the study, it will not affect your future medical care.

Please notify the study personnel as soon as possible if you wish to withdraw from the study. If you voluntarily withdraw from the study and you do not want your data to be used in the study, your samples will be destroyed.

Who Do I Contact if We Have Questions About the Study?

If you have any questions regarding your participation in this study, please feel free to contact Sandhya Roy (Research Co-ordinator) at 306-966-7851. Dr. Donna Chizen (Medical Investigator) at 306-966-8623, or Dr. Angela Baerwald (Scientific Investigator) at 306-966-8073. You may also contact the study investigators once the study is completed to enquire about the results of the study.

If you have questions about your rights as a research subject or concerns about the study and your experiences while participating in this study, you can contact the Chair of the Biomedical Research Ethics Board at the University of Saskatchewan at 306-966-4053. The Research Ethics Board is a group of individuals (scientists, physicians, ethicists, lawyers and members of the community) that provide an independent review of human research studies. This study has been reviewed and approved on ethical grounds by the University of Saskatchewan Research Ethics Board.

Consent to Participate

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- o I understand that by signing this document I do not waive any of my legal rights.
- o I will be given a signed and dated copy of this consent form.

I agree to participate in this study:

Participant Signature: _____

Participant Name (please print): _____

Date: _____ Time: _____

Investigator/Delegate Signature: _____

Investigator/Delegate Name (please print): _____

Date: _____ Time: _____

APPENDIX B

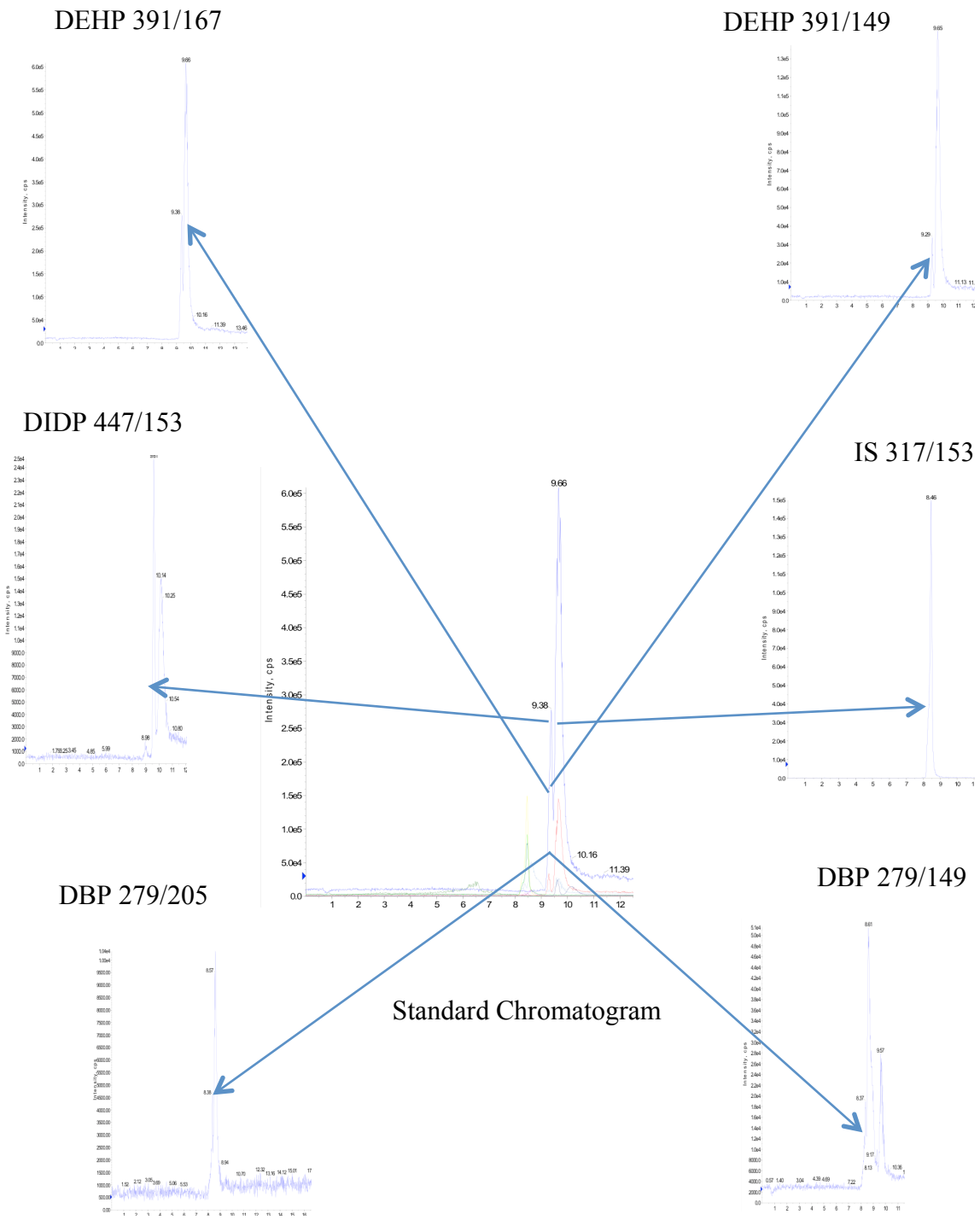

Figure 6.3.1: An example of a standard chromatogram. These graphs illustrate the data [run time in x axis (in min) and analyte peak area (an arbitrary unit) in y axis] which were used to identify one and/ or two products/daughter ions (i.e., DEHP, DBP, DIDP and internal standard in a standard) based on their retention time (RT). Arrows indicate the similar RT for two products ions of a precursor ion, and different RTs for each precursor ion.

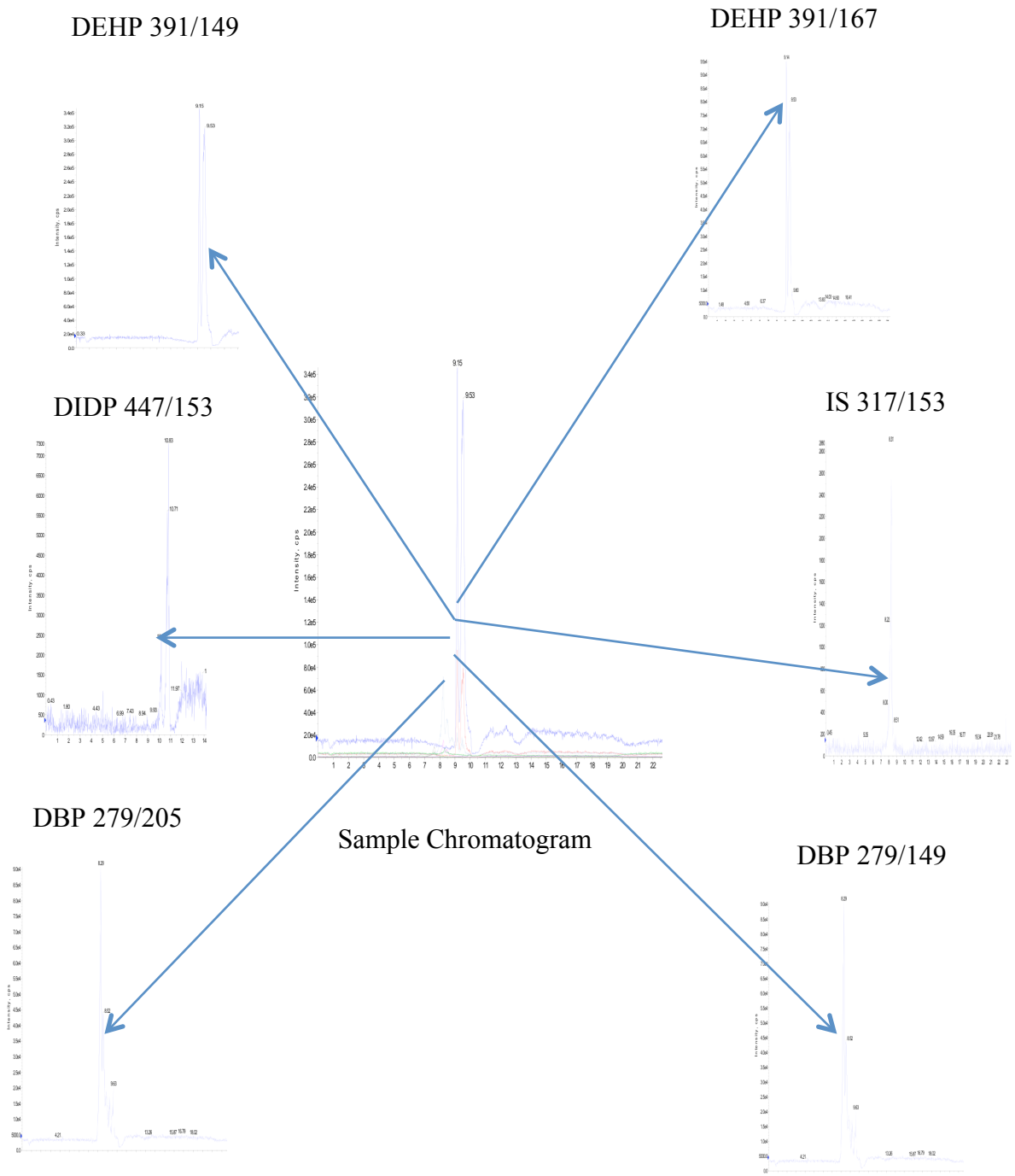

Figure 6.3.2: An example of a sample chromatogram. Graphs illustrate run time on the x axis (in min) and analyte peak area (an arbitrary unit) on the y axis. These data were used to identify one and/ or two products/daughter ions (i.e., DEHP, DBP, DIDP and internal standard in a standard) based on their retention time (RT). Arrows indicate the similar RT for two products ions of a precursor ion, and different RTs for each precursor ion.